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(54) Title: CUSTOMIZED OLIGONUCLEOTIDE MICROCHIPS AS MULTIPLE BIOSENSORS

(57) Abstract

　　Biosensors are constructed using customized micromatrices to design a microship. The microchip contains an arrangement of oligonucleotides designed to provide information on oligonucleotide sequences in a sample to be tested by the ability of the oligonucleotides in the nucleic acid sample to hybridize with oligonucleotides on the microchip. Biosensors are used to detect microorganisms in the environment, in biological samples and in food. Biosensors are also used to diagnose genetic defects, to identify polymorphisms, and to monitor quantitative gene expression.

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CUSTOMIZED OLIGONUCLEOTIDE MICROCHIPS
AS MULTIPLE BIOSENSORS

This invention relates to using customized
oligonucleotide microchips as biosensors for the
detection and identification of nucleic acids specific
for different genes, organisms and/or individuals in
5   the environment, in food and in biological samples.
"Environment" includes water, air and soil. Biological
samples include blood, skin, tumors amniotic fluid,
tissues, cells and cell cultures. Detection of
sequences in nucleic acids is used to identify
10   microorganisms in a sample, to diagnose genetic defects
or polymorphisms, to detect gene expression and for
forensic studies.
        Differences in nucleotide and amino acid
sequences may be exploited to analyze environmental,
15   food or biological samples. Detection and
identification of microorganisms is important for
clinical purposes and for determination of contaminated
food, air, water or soil. Studies in environmental
microbiology are often limited by the inability to
20   unambiguously identify and directly quantify the
enormous diversity of natural populations. This
problem is now changing with increasing use of
molecular techniques to directly measure different

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genetic features. (Mobarry et al., 1996; Stahl, 1995;
Wagner et al., 1995) For example, DNA probes are now
commonly used to detect by hybridization, genes
encoding proteins involved in specific catabolic

5   functions, and to resolve different genetic populations
in the environment. In particular, the use of group-
specific DNA probes complementary to the small subunit
(SSU) 16S rRNA has provided a comprehensive framework
for studies of microbial population structure in

10   complex systems. Sequencing of this subunit
revolutionized microbial classification and led to the
discovery of archebacteria. (Woese, 1987) A large
number of the sequences for different organisms has
been collected. (Maidak et al., 1996) Every

15   microorganism species is characterized by a specific
DNA sequence within a variable region of its ribosomal
RNA gene or other genes. A highly efficient procedure
for microorganism classification and for construction
of their evolutionary trees is based on these

20   observations. Identification of specific sequences in
ribosomal DNA is a reliable microbial analysis that can
be carried out by direct DNA sequencing. However DNA
sequencing is a rather complicated, expensive and time
consuming procedure to use for serial microbial

25   analysis on a commercial scale for environmental or
medical applications. Consequently, new methods are
needed to make sequence matching commercially feasible.

              A nucleic acid hybridization is a highly
specific and sensitive procedure that allows a specific

30   sequence to be detected and identified among other
millions of sequences in a genome of higher organisms,
or among a mixture of different organisms. The
principle of hybridization is that sequences hybridize
as a function of the similarity of their linear

35   nucleotide sequence. The hybridization of DNA or RNA
extracted from even a very complicated mixture to a

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specific oligonucleotide probe has resulted in
unambiguous identification of specific microorganisms
in an environmental sample, for example. In the course
of such an analysis, RNA or DNA is extracted from a

5   sample of microorganisms isolated from water solutions,
air or soil, immobilized on a filter and then
hybridized successively with several oligonucleotide
probes for different microorganisms. However, for this
purpose, the sample needs to be checked for the

10   presence of hundreds or thousands of different
oligonucleotides corresponding to various
microorganisms which is prohibitively laborious and
expensive using present methods.

         The scope of applications of nucleotide

15   hybridization is often limited by the nature of the
assays, generally involving the independent
hybridization of multiple environmental samples to
multiple DNA probes. In addition, some detection
assays require amplification of the target nucleic

20   acid, for example, via PCR. This may contribute to
quantitative biases. Thus, there is need for assays
that provide for greater sample through-put capacity
and greater sensitivity.

         Another area in which specific DNA or RNA

25   sequences are of interest is mutation and polymorphism
analyses. The number of base changes discovered
(mutations) in different genes is growing rapidly.
These changes are associated with genetic diseases,
with disease predispositions and cancers, with develop-

30   ment of drug resistance in microorganisms, and with
genetic polymorphisms. Polymorphisms are useful for
determining the source of a sample, e.g. in forensic
analyses. Polymorphisms such as in the HLA system are
essential to predict success of tissue transplants.

35   The ability to simultaneously analyze many mutations in
a gene in a simple, fast, and inexpensive way is
essential in clinical medicine and this need has

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stimulated the development of different methods for
screening mutations, but all have serious limitations.

    Hybridization of filter-immobilized DNA with
allele-specific oligonucleotides was suggested as a way
5   to screen for mutations. (Conner et al., 1983) How-
ever, the number of alleles that can be assayed at one
time is limited, the filters are usable only for a few
times, and there is little opportunity for complex
analysis.

10   A possible solution to large scale
hybridization is to use microchips for DNA sequence
hybridizations (SHOM, sequencing by hybridization with
oligonucleotides in a microchip) (e.g. Khrapko, 1996;
Yershov, 1996). The development of an array of
15 hundreds or thousands of immobilized oligonucleotides,
the so-called "oligonucleotide chips", permits
simultaneous analysis of many mutations (for a review,
see Mirzabekov, 1994). Such arrays can be manufactured
by a parallel synthesis of oligonucleotides (Southern
20 et al., 1992; Fodor et al., 1991; Pease et al., 1994;
Matson et al., 1995) or by chemical immobilization of
presynthesized oligonucleotides (Khrapko et al., 1991;
Lamture et al., 1994; Ghu et al., 1994). Glass
surfaces (Southern et al., 1992; Fodor et al., 1991;
25 Ghu et al., 1994), glass pores (Beattie et al., 195),
polypropylene sheets (Matson et al., 1995), and gel
pads (Khrapko et al., 1991; Yershov et al., 1996) have
been used as solid supports for oligonucleotide
immobilization. However "Oligonucleotide array
30 technology has not yet lived up to its promise."
Southern, 1996 p. 115. Some of the deficiencies are
unpredictability of the results, lack of knowledge of
optimum conditions, and failure to demonstrate accuracy
and commercial feasibility.

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SUMMARY OF THE INVENTION

This invention embodies applications of oligonucleotide microchip technology wherein the microchip is a biosensor and customized oligonucleotide microchips are designed for specific applications of nucleic acid hybridization. Hybridization is a process by which, under defined reaction conditions, partially or completely complementary nucleic acids are allowed to join in an antiparallel fashion to form specific and stable hydrogen bonds.

Aspects of the invention include improved predictability, increased accuracy, and standardized factors for detection and identification of nucleotide sequences. The improvements result from optimizing conditions, methods and compositions for microchip hybridization. Ordered schemes are followed so that much information can be obtained from a single scan of a microchip to detect hybridization of oligonucleotides in a sample to be investigated. Samples include air, water, soil, blood, cells, tissue, tissue culture and a food. An aspect of the invention is that the same microchip can be used for hybridization for more than 20-30 times, without any noticeable deterioration of the hybridization signal. Customized sets of micro-chips are obtained for specific applications. Also, parallel hybridization of nucleic acids in a sample to many oligonucleotides on a microchip is possible, allowing replication and standardization. For example, the sequence diversity of SSU rRNAs recovered from different microbial populations of varying abundances is analyzed by a single hybridization to a microchip. A large number of HLA alleles, are assayed by a single hybridization to a microchip.

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The invention relates a method for identifying a nucleotide sequence in a sample using a microchip, said method comprising:

a)   extracting nucleic acids from said

5   sample;

b)   providing a customized matrix of oligonucleotides on the microchip designed to identify the sequence in the sample;

c)   hybridizing said extracted nucleic acids

10   as such or after amplification on said microchip; and

d)   identifying the nucleotide sequence represented in said sample by comparing the identity of the oligonucleotides which hybridized to the sequence and the oligonucleotides not hybridized.

15   The nucleic acids include DNA, mRNA, 16S rRNA sequences and other RNA species.

Customized oligonucleotide microchips are aspects of the invention. The microchip includes a gel-matrix affixed to a support, said matrix is formed

20   by a plurality of gel pad element sites. The number of sites is determined by the number of oligonucleotides in the array. Each gel element contains one chemically immobilized oligonucleotide of a desired sequence, length and concentration; the gel elements being

25   separated from one another by hydrophobic glass spaces and the gel portions having a vertical height above the plane of the interstitial spaces of generally not more than 30 μm.

The invention relates screening nucleic acid

30   preparations for genes, RNA transcripts or any other unique nucleotide sequences, for example those that encode microbial 16S ribosomal RNAs. Ratios of DNA/RNA or any other unique nucleotide sequences specific for certain types of organisms are suitable. Multiple

35   labeling allows simultaneous detection and quantitative

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comparison of different nucleic acid sequences that are
hybridized to a microchip.

The methods of the present invention include
labeling the oligonucleotide sequence in said sample
5   before bringing it in contact with the array. A suit-
able label is a fluorescent dye. A plurality of dif-
ferent dyes may be used concurrently. Oligonucleotides
on a customized microchip include those complementary
to the beta globin gene, sequences specific for
10   *Salmonella*, or polymorphic HLA allele sequences.

An oligonucleotide microchip for the
detection and classification of nitrifying bacteria has
a customized design wherein identifying labels in the
cells of the microchip refer to oligonucleotides
15   selected from a class of bacteria, and the selection is
designed to answer specific questions regarding
classification.

An embodiment of an application of the
present invention is detecting and identifying
20   microorganisms in samples obtained from the
environment, e.g. water, air or soil samples to check
for pollutants; biological samples obtained for medical
diagnosis; or food samples to check for contamination.
Other applications include forensic testing to identify
25   DNA in samples obtained for criminal investigations,
and detection of chromosomal fragments, or single gene
mutations e.g. for diagnosing genetic diseases such as
β-thalassemia or types of cancers. Tissue typing for
polymorphic HLA alleles for transplantation or studying
30   human diversity is facilitated.

The nucleic acid preparations are made from
samples collected in any type of environment, where
detection and identification of the microorganisms in
that environment is of interest, or where it is likely
35   that new (previously unidentified) organisms may be
discovered. The volume of each microchip is great

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enough to accommodate a high surface concentration of
probes, allowing a large dynamic range for measurement.

        DNA and RNA molecules in a sample can be
separated from each other during their isolation and
labeled with different fluorescent dyes. These RNA and
DNA molecules are simultaneously hybridized with
oligonucleotides on a microchip that is specific to the
sample to be tested. The quantitative monitoring of
the simultaneous hybridization of differently labeled
DNA and RNA with a microscope that can discriminate
multicolors at several wave lengths allows the
calculation of DNA/RNA ratios in the sample. For
bacterial samples, this ratio determines the state of
vitality and physiological activity of the bacterium.
In an embodiment, the ratio of RNA/DNA is used to
discriminate the dead bacterium cells and spores from
the active state of microbial growth. In the same way,
a DNA or RNA molecule of a bacterial strain stained
with one dye can be added in a calculated amount as an
internal standard to a sequence or sequences under
investigation in which the sequences being investigated
stained with a different (second) dye. The
fluorescence measurements of hybridization intensities
at different wave lengths for the standard and
investigated sequences (probes) allow relative
quantitative ratios to be determined.

        Hybridization on microchips allows
unambiguous typing of different groups of chosen
bacteria in a sample. Microchip hybridization is a
simple, fast, inexpensive and reliable method for
bacterial typing.

        An aspect of the invention is that there is
no limitation on the number of sequences that can be
checked or the number of types of microorganisms that
can be detected. Instead of multiple sequential
hybridizations with different probes of, e.g. a 16S

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rRNA preparation, only one round of hybridization is required to find out what different sequences are in a sample. The volume of hybridizations is dramatically reduced and the assay requires much less RNA or DNA

5   compared with standard techniques. An advantage is that culturing of bacteria and gene amplification can be avoided.

Methods of the invention significantly reduce sample preparation time, avoid the culturing of

10 organisms collected from field situations, and allow the identification of all species of microorganisms contained in a particular sample.

For example, oligonucleotides complementary to small subunit rRNA sequences of selected microbial

15 groups, encompassing key genera of nitrifying bacteria, were shown to selectively retain labeled target nucleic acid derived from either DNA or RNA forms of the target sequences. Methods and compositions of the present invention discriminate among the Genera, *Nitrosomonas*,

20 *Nitrobacter* and *Nitrosovibrio sp.* using fluorescently labeled nucleic acid probes that hybridize to 16S rRNA sequences. Each species has specific DNA sequences within the variable region of its rRNA genes. Since the rRNAs are naturally amplified, often present in

25 thousand of copies per cell, they provide greater sensitivity, eliminating the need for amplification in many applications.

The invention facilitates identification of organisms from environmental samples in a faster, and

30 more economical approach than presently available. In addition, new species may be discovered that would be highly informative regarding taxonomic status of known as well as newly discovered organisms.

A diagnostic assay of the present invention

35 for a mutation in a gene, includes the following steps:

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     a.   designing a customized oligonucleotide microchip biosensor comprising oligonucleotides that hybridize to a gene having the mutation;

     b.   contacting a nucleic acid sample to the customized oligonucleotide microchip biosensor under conditions that allow hybridization of the nucleic acid to the microchip; and

     c.   determining whether hybridization occurs from which observation the presence of the specific nucleic acid sequence is inferred.

For diagnostic assays for genetic diseases, sequence analysis of DNA is carried out by hybridization of PCR amplified DNA or its RNA transcripts with oligonucleotide array microchips. Polyacrylamide gel pads containing allele-specific immobilized oligonucleotides are fixed on a glass slide of the microchip. The RNA transcripts of PCR-amplified genomic DNA are fluorescently labeled by enzymatic or chemical methods and hybridized with the microchip. The simultaneous measurement in real time of the hybridization and melting curves on the entire oligonucleotide array is carried out with a fluorescence microscope equipped with CCD camera. The monitoring of the hybridization specificity for duplexes with different stabilities and AT content is enhanced by its measurement at optimal discrimination temperatures on melting curves. Microchip diagnostics are optimized by choosing the proper allele-specific oligonucleotides from among the set of overlapping oligomers. The accuracy of mutation detection can be increased by simultaneous hybridization of the microchip with at least two differently labeled samples of normal and mutated alleles, and by parallel monitoring their hybridization with a multi-wavelength fluorescence microscope. The efficiency and reliability of the sequence analysis was demonstrated

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by diagnosing β-thalassemia mutations and HLA polymorphisms.

Determining levels of gene expression is an aspect of the invention.

5   Because the methods of the present invention require only a simple procedure of hybridization and because only one round of hybridization is necessary, it is fast and inexpensive. Because the invention allows a lot of information to be obtained from one

10   experiment, it is efficient. The invention is reliable because the microchips are reusable. There is no waste of hybridization probes, therefore the microchip hybridization is inexpensive and non-isotopic detection simplifies all procedures.

15   Effective and precise sequence analysis by the hybridization of a probe with rather short microchip-immobilized oligonucleotides depends on many factors. Major factors are the reliability of the discrimination of perfect duplexes from duplexes

20   containing mismatches, differences in stability of AT- and GC-rich duplexes, the efficiency of the hybridization, and simplicity in the preparation of the labeled samples for hybridization.

Identification of base variations is

25   significantly improved by parallel measuring of the melting curves of the duplexes formed on the entire oligonucleotide array, as well as by monitoring the simultaneous hybridization of two differently labeled samples at two wavelengths and by choosing proper

30   allele-specific oligonucleotides.

Other factors to be considered for operation of the invention include (1) regulating the flow of the fluid containing a sample to be tested over the micro- chip during the hybridization; and (2) control of the

35   temperature of the microchip gel layer and the fluid layer, in a differential manner, by placing a cooling

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and heating apparatus adjacent to the gel layer and the top fluid layer. The gel layer temperature is controlled in a uniform or gradient manner by a heating/cooling device attached to the glass plate

5   substrate of the gels.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows non-equilibrium melting curves of duplexes of RNA with microchip oligonucleotides.

10   FIG. 2 shows an example of four melting curves for 75-nt-long RNA fragments hybridized with the microchip oligonucleotides. The RNA was derived from a patient having the IVS I/2 T/A mutation in the β-globin gene. The curves were normalized to the initial

15   hybridization signals. Melting curves 1 and 3 correspond to perfect duplexes; curves 2 and 4 correspond to duplexes containing internal T-T or G-T mismatches, respectively. The curves for the perfect and mismatched duplexes are shifted by about 10°C from

20   each other.

FIG. 3 shows hybridization of fluorescein labelled 16S rRNAs to a microchip. The microchip with immobilized probes (see Table 1 and Table 2) was hybridized sequentially to *in vitro* transcribed 16S

25   rRNA of *Nitrosovibrio tenuis* (A), *Nitrosomonas europaea* (B), *E. coli* (C), and with *E. coli* rRNA recovered from isolated ribosomes (D). The panels to the right display the number of mismatches between each probe and the RNA.

30   FIGS. 4A and 4B show hybridization of the mixture of differently labelled *E. coli* and *Nitrosovibrio tenuis* rRNAs to the microchip at 10°C and 40°C, measured simultaneously by multicolor detection.
A. The microchip was hybridized with a mixture of

35   fluorescein labelled *Nitrosovibrio tenuis* and

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tetramethylrhodamine labelled *E. coli* 16S rRNA and washed serially at the indicated temperatures, a.u. - arbitrary units of fluorescence intensities. B. The ratio of the hybridization intensities of *Nitrosovibrio*

5 *tenuis* (I_{Nt}) to *E. coli* ($I_{E.coli}$) 16S RNA measured at 10°C and 40°C. $R = (I Nt / I_{E. coli})$.

FIG. 5 illustrates the concentration effect of the immobilized oligonucleotides on the hybridization intensities. A microchip with different

10 concentrations of immobilized oligonucleotides was hybridized with *N. tenuis* 16S rRNA labelled with fluorescein and washed at 20°C. Curve 1 corresponded to Nsv443 (nitrosovibrio-like) probe, curve 2 - Bac338 (Bacteria), curve 3 - Nso1225 (ammonia oxidizers),

15 curve 4 - Uni1390 (all life), and curve 5 - Nsm-156 (nitrosomonas), a.u. - arbitrary units of fluorescence intensities.

FIG. 6 shows the sequences of β-globin alleles specifying oligonucleotides that were

20 immobilized on a microchip.

FIG. 7 shows the experimental design to detect β-globin mutations using oligonucleotide microchips.

FIG. 8 shows results of gene expression

25 studies.

FIG. 9 shows 18 short HLA oligonucleotides.

FIG. 10 shows HLA oligonucleotides hybridized to the microchips.

30 ### DESCRIPTION OF THE PREFERRED EMBODIMENT

This invention relates to using customized oligonucleotide microchips as biosensors for the detection and identification of nucleic acids specific for different genes, organisms and individuals in the

35 environment, in food and in biological samples.

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"Environment" includes water, air and soil. Biological
samples include blood, skin, tumors amniotic fluid,
tissues, cells and cell cultures. Detection of
sequences in nucleic acids is used to identify
5   microorganisms in a sample, to diagnose genetic defects
or polymorphisms, to detect gene expression and for
forensic studies.

A nucleic acid hybridization is a highly
specific and sensitive procedure and allows a specific
10   sequence to be detected and identified among other
millions of sequences in a genome of an organism.
However, nucleic acid hybridization is a useful but
quite a cumbersome procedure. This drawback can be
overcome by using oligonucleotide microchips as
15   biosensors for different microorganisms. Within a
small area of a few square millimeters or centimeters,
hundreds and thousands of synthetic oligonucleotide
probes are immobilized that are specific to ribosomal
DNA or to other specific nucleic acids. Subsequent
20   hybridization of a DNA or RNA molecule to the microchip
enables a menu of oligonucleotides to be identified in
a sample. For bacterial assays, pure culture
microorganisms, purified target nucleic acid or even
synthetic oligonucleotides are useful as internal
25   standards, serving to estimate the efficiency of
nucleic acid isolation or the absolute amount of target
nucleic acid recovered.

The customized oligonucleotide microchips are
produced by chemical immobilization of presynthesized
30   oligonucleotides, or by direct synthesis of
oligonucleotides on a microchip. If a microchip
contains rather long oligonucleotides, the former
methods are the methods of choice because before
immobilization, the oligonucleotides are purified and
35   checked for their quality.

Methods and technologies have been developed
for microchip manufacturing, hybridization of

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fluorescently labeled DNA and RNA with the microchips
and monitoring the hybridization with a fluorescence
microscope equipped with CCD-camera, computer and
proper software (see U.S. Pat. No. 5,552,270 herein
5   incorporated by reference).

The oligonucleotide microchips consist of
many polyacrylamide gel pad elements generally of the
size of 40x40x20 μm and larger. The elements are
chemically fixed on a glass surface. Each microchip
10   gel element contains a specific presynthesized
oligonucleotide that is immobilized through a covalent
bond. Hundreds of microchips containing hundreds and
thousands of different immobilized oligonucleotides can
be manufactured by a specially devised robot. The gel
15   array also offers several advantages over formats using
an *in situ* synthesis of the oligonucleotide array. The
synthetic oligonucleotides are purified by gel electro-
phoresis or HPLC prior to immobilization on the micro-
chip. This provides for stringent quality control of
20   oligonucleotide purity and insures high specificity.
The polyacrylamide gel support has a capacity of
immobilized oligonucleotides from 0.03 pmol up to 10
pmol per 100x100x20 μm gel pad. This offers improved
quantification and better discrimination between
25   perfect and mismatched duplexes. It also provides a
way to normalize differences in hybridization signal
intensities.

Oligonucleotide microchip technology for
sequencing by hybridization is available to identify
30   the presence of microorganisms in a sample of any type,
or to find new species. As shown in the examples
herein, the hybridization of DNA or RNA extracted from
even a very complicated mixture to a specific
oligonucleotide probe has resulted in unambiguous
35   identification of microorganisms. The nucleotide
sequence of the microorganisms for genes encoding a

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small subunit of ribosomal 16S rRNA forms the basis for
a microchip biosensor. Instead of direct sequencing of
the gene, hybridization analysis of DNA or RNA samples
with oligonucleotides specific for the microorganisms
5   is performed. This new technology provides efficient
microbial analysis and environmental monitoring.
Fluorescently labeled DNA and RNA samples from
microorganisms are hybridized with microchips
containing oligonucleotides specific for several
10   microorganisms. These microorganisms are reliably
identified by microchip hybridization. Microorganism
biosensor technology is developed, customized microbial
oligonucleotide microchips are produced by methods of
the present invention, and methods are developed for
15   simultaneous quantitative and qualitative microchip
analysis of hundreds and thousands of microorganisms in
a sample and for discovery of new ones.

Other applications include detection of
genetic mutations such as are characteristic of hemo-
20   globin disorders; detection of genetic polymorphisms
such as HLA; investigation of gene expression;
detection of causative agents of diseases; forensic
studies; and detection of microbial pollutants.

25   **EXAMPLES**

The following examples are presented as
illustrations of aspects of the invention, rather than
limitations on the invention.

Example 1: **Preparation of an Oligonucleotide**
30   **Microchip Biosensor**

Oligonucleotides are synthesized using a 394
DNA/RNA synthesizer (Applied Biosystems). The
synthesis of oligonucleotides for immobilization began
with 3-methyluridine at the 3'-terminal position.

35   In one embodiment, fluorescently labeled RNA
was prepared using T7 RNA polymerase. Template DNA

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(133 and 75 bp long) for in vitro transcription was prepared by PCR amplification with the nested primers T7V2L-45, 5'-GGAATTCCTAATACGACTCACTATAGGGACACCATGGTGCACCTGACTCC-3'

5   (SEQ ID:1), as well as with the common reverse primer T7-V2L-103 5'-GGAATTCCTAATACGACTCACTATAGGGAGGTGAACGTGGATGAAGTTGG-3'(SEQ ID:2) and 5'-TCTCCTTAAACCTGTCTTGTAACC-3' (SEQ ID:3). Templates were purified using a QIAquick PCR

10   purification kit (QIAGENE) according to the manufacturer's protocol. The RNA polymerase reaction was performed using the MEGAshortscript™ T7 kit (Ambion) with fluorescein 12-UTP (Molecular Probes). Fluorescently labeled ssDNA (single stranded DNA)

15   fragments were prepared by single primer reamplification.

A polyacrylamide gel micromatrix was prepared by photopolymerization of a solution of 4% acrylamide (acrylamide/bisacrylamide 19/1), 40% glycerol, 0.0002%

20   methylene blue, and 0.012% TEMED in 0.1 M sodium-phosphate buffer, pH 7.0. The mixture was applied to an assembled polymerization chamber illuminated with U.V. light.

Two types of microchip matrices (micro-

25   matrices) were routinely prepared with gel pad elements of about 60x60x20 μm and 100x100x20 μm that were spaced by 120 and 220 μm, respectively. About 1 nl of activated oligonucleotide solution was transferred to a gel element using either a robot or a simple manual

30   device.

The device includes a Peltier thermostated pin placed under a binocular lens in conjunction with a micromanipulated holder, a power supply, an a refrigerated circulator.

35   The manufacture of microchips of gel-immobilized oligonucleotides basically consists of

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three steps; shaping the desired topology of oligo-
nucleotides on a gel micromatrix; loading microvolumes
of oligonucleotide solutions onto the micromatrix, and
immobilizing within the gel oligonucleotides containing
5   the active 3' or 5' terminal aldehyde or amine groups.

To avoid the exchange of different
oligonucleotide solutions applied on adjacent gel pads,
the pads are separated on the micromatrix by a
hydrophobic glass surface. Two-dimensional scribing or
10   laser evaporation is used for micromatrix preparation,
but these procedures require rather complex equipment
and experienced personnel. The photopolymerization
method significantly simplifies the procedure and makes
it accessible to a biochemical laboratory.

15   Microfabrication by mask-directed
photopolymerization (e.g., a photoresist method in
microelectronics) is a well developed technique. From
several acrylamide photopolymerization techniques
tested, modified - methylene - blue induced photo-
20   polymerization produced the best results for
micromatrix manufacture. The gel matrix consists of
gel pads photopolymerized on a glass slide. The gel
pads are formed according to the mask topology due to
the lack of photopolymerization in places covered by a
25   nontransparent grid.

The microchip is manufactured by applying the
activated oligonucleotide solutions onto the micro-
matrix of gel elements containing active hydrazide or
aldehyde groups. A simple device exists for manual
30   loading of up to 100 different oligonucleotides on a
micromatrix. The transfer is carried out by the
hydrophilic upper surface of a pin that is first
immersed into, and is wetted with, an oligonucleotide
solution, and then is withdrawn from the solution and
35   brought into contact with the gel surface. This
transfers about 1 nl of oligonucleotide solution with a

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reproducibility of ±10%. The temperature of the pin is maintained near the dew point of the ambient air to avoid the evaporation of this microvolume solution in the course of transfer.

5　　　　**Example 2**: **The hybridization of microchips with DNA and RNA**

Fluorescently labeled DNA or RNA (5 μl, 0.1-1 pmol/μl were hybridized to a microchip at +5°C in a hybridization buffer containing 1 M NaCl, 1 mM EDTA, 1%

10　Tween-20, and 10 mM sodium phosphate at a pH of 7.0, for between about 2-24 h. The microchip was covered with a cover glass or a Teflon sheet so that a 300-μm space is above. Then the hybridization solution containing DNA or RNA fragments was substituted with 10

15　μl of cooled hybridization buffer. The microchip with the cover glass was placed on a thermostabilized table. Hybridization was monitored quantitatively using a specially constructed multicolor epifluorescent microscope with a 4x4 mm observation field equipped

20　with a CCD camera and suitable software.

Example 3: **Analysis of Melting Curves**

The polyacrylamide gel used on a microchip provides more than 100 times higher capacity for three-dimensional immobilization of oligonucleotides than

25　does a two-dimensional glass surface. The high concentration of immobilized oligonucleotides facilitates the discrimination of mismatched duplexes and enhances the sensitivity of measurements on the microchips. This allows the use of a CCD-camera-

30　equipped fluorescence microscope (Yershov *et al.*, 1996) although it is less sensitive than laser scanning systems (Lipshutz *et al.*, 1995), but offers the advantage of monitoring the hybridization on a microchip at different temperatures in real time for

35　measurement of the melting curves. Melting curves are

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defined herein as produced by plotting the amount of
duplexes [fluorescent intensity] versus temperature.
The procedure, the software, and the hybridization
microchamber (Yershov et al., 1996) have all been

5   developed for recording melting curves at a wide range
of temperatures simultaneously for perfect and
mismatched duplexes formed upon hybridization of a
probe with all microchip oligonucleotides.

A significant amount of time is needed for

10   the microchips hybridized with rather long RNA or DNA
probes to achieve equilibrium. Therefore, non-
equilibrium dissociation melting curves were measured.
However, they are not far away from equilibrium where
some difference in heating rate did not significantly

15   affect the results. The melting curves for hybridiza-
tion of, for example, synthetic 19-mers with the
microchip oligonucleotides reached equilibrium under
the same conditions that were used for measuring non-
equilibrium RNA and DNA melting curves. The melting

20   curves can also be measured after a few minutes, far
away from equilibrium, if an internal standard is added
to a tested sample. This standard can be a differently
labeled RNA of a normal allele. This significantly
speeds up the identification of nucleic acid base

25   changes.

Example 4: Choice of Optimum Melting

Temperatures.

This invention embodies an improvement in the
SHOM technology in which hybridizations between an

30   array of gel-immobilized nucleotides (a microchip) and
the unknown nucleotides to be tested are measured at
optimal, discriminatory melting temperatures. This
improvement is achieved by parallel measuring of the
melting curves of the duplexes formed by hybridization

35   on the entire oligonucleotide array, as well as by
monitoring the simultaneous hybridization of two

samples of nucleotides labeled with different
fluorochromes, and judicious choice of proper allele-
specific oligonucleotides as the immobilized probes.
The fluorochromes chosen for the labeling emit light of
5   sufficiently differing wavelengths, that both types of
labels can be measured in the same reaction mixture.

The greatest discrimination between perfect
and mismatched duplexes was achieved at a temperature
at which the intensity of the hybridization signal from
10   a perfect duplex dropped to one-tenth of its initial
value; at such a temperature, the hybridization
intensities from mismatched duplexes usually approached
the background level. The temperature at which the
initial signal of hybridization drops by a factor of 10
15   is termed the discrimination temperature (Td.).

In the case of beta-thalassemia mutation
detections described in Example 6 herein: (1) RNA
transcripts of PCR-amplified DNA were hybridized with
immobilized oligonucleotides; (2) the Td values for
20   perfect 40% and 70% GC-rich duplexes were 52° and 64°,
respectively; (curves 1 and 3 in FIGS. 1A and 1B);
(3) the immobilized oligonucleotides were chosen from
among a set of overlapping sequences; and (4) the two
samples included in the reaction mixture were a mutated
25   allele RNA labeled with one fluorochrome and a sample
of the normal allele RNA labeled with a different
fluorochrome.

The Td is determined by hybridization with an
RNA sample if an allelic DNA is available. If such DNA
30   is unavailable, the Td can be measured from the
hybridization data resulting from experiments performed
with synthetic oligonucleotides corresponding to the
mutated allele of interest.

The dissociation curves for perfect and
35   mismatched duplexes are parallel at the range of about
10° (in the middle of the curves) when plotted on a

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semilogarithmic scale. At this 10°C range, the ratios
of the signals for perfect and mismatched duplexes
remain rather constant. This makes the discrimination
procedure robust to some inaccuracies in determining

5   Td. The discrimination temperature depends on
experimental conditions (rate of heating, ionic
strength, probe concentration, extent of fragmentation,
and so forth) which can vary from one experiment to
another. However, these variations affect Td and the

10   relative intensities of the hybridization signals to a
similar extend for all microchip elements and therefore
do not significantly distort the discriminations.
Therefore, to provide a reference Td, the
oligonucleotides CD26(N) and CD 26 G/A, which form

15   perfect and mismatched duplexes, respectively, with all
RNAs tested, were introduced into the microchip.

        Since Td is robust to some inaccuracy in
measurements, 19-mer oligodeoxynucleotides were used in
these experiments instead of more expensive 19-mer

20   oligoribonucleotides. There are differences in the
stability of DNA-DNA homoduplexes relative to DNA-RNA
heteroduplexes (Lesnik and Freier, 1995). The pattern
of hybridization of the microchip with RNA derived from
patients and with 19-mers was rather similar to that

25   from the 10-mers. Hybridization with corresponding
synthetic oligonucleotides is preferred as a control
when a mutation is identified in an RNA sample by its
hybridization with a diagnostic microchip.

        A mixture of fluorescently labeled RNA

30   samples was prepared from two patients; the first
sample was TMP-labeled RNA from a patient that is
homozygous for the normal CD26 area of the beta-globin
allele; the second sample is fluorescein-labeled RNA
from a patient that is heterozygous for the normal CD26

35   area and a mutation CD26 G/A alleles. This mixture was
hybridized with a microchip consisting of two microchip

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elements that contained the following immobilized oligonucleotides:

SEQUENCE

A sample (element)-CD26(N-normal)   5'-GGCCT<u>C</u>ACCA-3MeU-3' (SEQ ID:4)

5   B sample (element)-CD26(G/A-mutant)   5'-GGCCT<u>T</u>ACCA-3MeU-3' (SEQ ID:5)

The underlined bases indicate the position of a nucleotide change between the two samples.

Usage of different filters during the
10   registration of the signal, allowed the independent, simultaneous registration of the sample, which was marked with the different dyes; TMP (red) and fluorescein (green), on the same element of the microchip. FIG. 2 demonstrates the interaction of
15   sample 1 with the A microchip element; Graph 2 demonstrates the interaction of sample 2 with the A microchip element; Graph 3 demonstrates the interaction of sample 2 with the B microchip element; and Graph 4 demonstrates the interaction of sample 1 interaction
20   with the B microchip element.

Example 5: Use of a Customized Microchip Matrix Biosensor to Identify Nitrifying Microorganisms.

Microorganisms that degrade nitroaromatic compounds include Pseudonomas, Arthrobacter, Nocarida,
25   Myco-bacterium, and fungi.

Previously, methods for detection of these bacteria were tedious and inaccurate. For example, to detect Pseudonomas capable of degrading nitroaromatic compounds, 2-nitroluene was tested as a sole carbon,
30   energy and nitrogen source. It was difficult to isolate the bacteria from soil samples to perform the test.

Nitrifying bacteria have proved particularly difficult to study using cultivation techniques, such
35   as most probable number (MPN) and selective plating because of their long generation times and poor

-24-

counting efficiencies. Thus, a rapid and non-culture dependent enumeration technique for nitrifiers could greatly facilitate research in their ecology.

Microchips with 100x100x20 μm gel pads

5   (alternatively 60x60x20 μm), fixed on a glass surface and containing a set of 10 oligonucleotides 15-20 bases-long were manufactured for bacterial typing experiments. The set included oligonucleotides complementary to different regions of 16S ribosomal

10   RNA. Since rRNA's are naturally amplified, and often are present in thousands of copies per cell, they provide great sensitivity and eliminate the need for amplification in many applications. One oligonucleotide is represented in most living

15   organisms, another is typical for most of bacteria and the rest belong to nitrosos (nitrifying) bacteria only. The group of nitrosos bacteria oligonucleotides consists of two oligonucleotides typical of nitrobacter, two typical of nitrosomonas and one

20   typical of nitrosovibrio. One oligonucleotide was complementary to an antisense strand of rDNA for hybridization with ribosomal dsDNA, that was PCR amplified from genomic or cDNA.

The following scheme for an ordered

25   oligonucleotide loading (placing on a chip) is useful for bacterial (or organism, species) typing. In the micromatrix design shown in Table 1, the first oligonucleotides characterize the highest order (i.e. to distinguish a living organism). [Uni 1390-CIII].

30   Reducing the order step by step down to the lowest level, i.e. from family, to genus, to species provides further discrimination of oligonucleotides that are present in a sample being investigated. For example, for oligonucleotides used to classify nitrifying

35   bacteria, a bacterial oligonucleotide would be in the next position. [Bac 338 - CI and NonBac338 - CII].

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Oligonucleotides specific to nitrobacter [Nb1000 - AI and NIT3 - AII] and ammonia oxidizers [NEU23 - AIII, Nso190 - AIV and Nso1225 - BI] follow in any order. Finally, oligonucleotides specific to Nitrosomonas [Nsm156 - BII] and Nitrosovibrio [Nsv443 - BIII] complete the micromatrix design.

The microchip was evaluated using three different rRNA preparations (phenol extracts of cellular RNA, RNA isolated from purified ribosomes, and *in vitro* transcripts of cloned ribosomal DNA), and both fragmented double-stranded and single-stranded DNA. Hybridizations were performed in a formamide buffer at low temperature in order to enhance microchip durability and decrease RNA degradation. Although all DNA and RNA preparations could be used, the best discrimination was observed for *in vitro* transcribed rRNAs using the hybridization conditions evaluated in this study.

The hybridization of the microbial microchips was carried out with five different preparations of target nucleic acids. Ribosomal RNA and total RNA were recovered from cells. RNA transcribed *in vitro* as well as single- and double-stranded PCR-amplified 16S rDNA were obtained from plasmids containing the cloned 16S rRNA gene. All of these sample types provided a comparatively reliable identification of the microorganisms by their hybridization with the microchip-immobilized oligonucleotides and could be used for different purposes. For example, the rRNA provides a naturally amplified target. Also, since cellular ribosome content is well known to vary with growth rate, it is generally thought that direct quantification of rRNA serves to identify the more active environmental populations. In contrast, analysis of PCR amplified rDNA provides a more general measure of all microorganisms present in a sample. Alternatively, these measures could be combined. For

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example, the RNA and DNA components of an environmental
sample could be isolated and labelled with different
fluorescent dyes. Following their combined
hybridization, the resulting ratio of RNA and DNA
5   hybridizing to an individual gel element could be used
to infer the physiological status of the corresponding
microbial population.

Table 2 shows the sequences of the
oligonucleotides and other characteristics of them.

10

TABLE 1

Micromatrix Design for Nitrifying
Microorganisms.

	I	II	III	IV
A	Nb1000	NIT3		Nso190
B	Nso1225	Nsm156	Nsv443	
C	Bac338	NonBac338	Uni1390	

TABLE 2

Oligo-nucleotide Name and Position	Sequence (5' to 3')	Specificity	Microchip location Table 1	Td[1] °C
Nb1000	5'-TGC GAC CGG TCA TGG-3' (SEQ ID:6)	*Nitrobacter*	A-1	42°
NIT3	5'-CCT GTG CTC CAT GCT CCG-3' (SEQ ID:7)	*Nitrobacter*	A-II	66°[2]
NEU23	5'-CCC CTC TGC TGC ACT CTA-3' (SEQ ID:8)	Ammonia oxidizers	A-III	66°[2]
NSO190	5'-CGA TCC CCT GCT TTT CTC-3' (SEQ ID:9)	Ammonia oxidizers	A-IV	62°
NSO1225	5'-CGC GAT TGT ATT ACG TGT GA-3' (SEQ ID:10)	Ammonia oxidizers	B-I	51°
NSMO156	5'-TAT TAG CAC ATC TTT CGA T-3' (SEQ ID:11)	*Nitrosomonas*	B-II	46°
NSV443	5'-CCG TGA CCG TTT CGT TCC-3' (SEQ ID:12)	*Nitro-sospira*-like	B-III	52°
BAC338	5'-GCT GCC TCC CGT AGG GAT-3' (SEQ ID:13)	Bacteria	C-I	54°
NonBAC338	5'-'ACT CCT ACG GGA GGC AGC-3' (SEQ ID:14)	Eub338 complementary strand	C-II	54°
UNI1390	5'GAC GGG CGG TGT GTA CAA-3' (SEQ ID:15)	all life (with a few exceptions)	C-III	44°

[1] Experimentally determined.

[2] Estimated from *in situ* hybridization.

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A number of hybridization conditions were
tested in terms of efficiency and specificity of
hybridization. Hybridization in formamide containing
buffer at low temperature gave good results.
Hybridizations were performed at 5° centigrade in 33%
formamide. RNA samples and covalent bonding of
oligonucleotides with the support (hence durability of
microchips) are more stable at low temperatures. In
addition, these conditions were favorable from a point of
view of RNA stability and microchip durability similar to
other RNA molecules at low temperatures of about 0°-5°C.

The hybridization on a microbial microchip was
carried out with *in vitro* RNA transcripts of 16S rDNA of
different nitroso bacteria, total RNA extracts and
ribosomal RNA extracted form *E. coli* and *Desulfovibria
vulgurus* as well as PCR amplified double or single
stranded DNA of 16S rDNA.

The probes for ammonia oxidizing bacteria show
different discrimination specificity under different
conditions. FIG. 3 shows the fluorescence of individual
gel elements on the microchip following hybridization to
the 16S rRNAs of *Nitrosovibrio tenuis* (A), *Nitrosomonas
europaea* (B), and *E. coli*, either *in vitro* transcribed
(C) or recovered from isolated ribosomes (D). The same
microchip was used for each hybridization following
washing with distilled water. Each microchip was
routinely used for up to 20-30 hybridization experiments.
The appropriate pattern of hybridization was observed for
all gel elements shown, despite a significant difference
in dissociation temperatures (T_{ds}) previously determined
using membrane support hybridization (Table 1). For
hybrids of comparable stability, discrimination is
generally achieved by washing at increasing temperatures

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(described below) or by simultaneously evaluating their
melting characteristics, since the fluorescence analyzer
can monitor hybridization signals in real time.

FIGURE 4A and 4B shows the results of an
experiment evaluating the effect of increasing washing
temperature on target RNA retention. A mixture of
Nitrosovibrio tenuis and *E. coli* 16S rRNA labelled with
different fluorescent dyes (fluorescein and
tetramethylrhodamine, respectively) was hybridized to the
chip at 5°C. The hybridization solution was then
replaced with washing buffer and the retention of each
RNA species was measured following each 10°C incremental
increase in temperature (up to 60°C) using multicolor
detection. Nonspecific hybridization of *E. coli* rRNA to
Nso1225 (ammonia oxidizer), Nsm156 (nitrosomonas), and
NonBac338 (anti-sense) was observed following the 10°C
wash. However, this nonspecific hybridization was
significantly reduced following the 40°C wash. In like
manner, the 16S rRNA of *Nitrosovibrio tenuis* hybridized
to *Nitrosomonas* (Nsm156) at 10°C, but was reduced to near
background (compared to NonBac338) following the 40°C
wash. A more complete correction for differences in
stabilities of duplexes can be carried out by measuring
the equilibrium or non-equilibrium melting curves for all
microchip elements. This would provide a basis to
compensate for the various factors influencing individual
duplex stability, e.g., their length, GC-content, and
competition with secondary and tertiary structures in RNA
and DNA.

FIG. 4B shows the ratios of hybridization
intensities of fluorescein labelled *Nitrosovibrio tenuis*
to tetramethylrhodamine labelled *E. coli.* with different
microchip oligonucleotides at 10°C and 40°C (the ratios

are derived from the data presented on Figure 4A. These
ratios were not changed significantly for oligo-
nucleotides specific to bacteria and all living organisms
between 10°C and for more stringent conditions at 40°C.

5   However, the ratio is dramatically increased at 40°C
(compared to 10°C) for oligonucleotides specific to
ammonia oxidizers and nitrosovibrio. This increase
reflects the greater duplex stability of *Nitrosovibrio
tenuis* RNA with the complementary oligonucleotides

10   compared with *E. coli.* RNA. Although the nitrosomonas
ratio increases, the signal originating from each
labelled RNA is near background. This experiment
demonstrates that the inclusion of second dye-labelled
RNA, either isolated from cells or synthesized, could be

15   used as an internal standard for quantitative assessments
of hybridization patterns.
        Variable hybridization to the different gel
elements is the expected consequence of using a single
hybridization condition to evaluate an array of probes,

20   each having different kinetics of association and
dissociation. To some extent these difference can be
normalized by varying the concentration of
oligonucleotides in the individual gel elements. For
example, the relatively low hybridization signals of

25   Nso1225(b-I) and Uni1390 (c-III) compared to Nsv443
(b-III) could each be elevated by increasing the amount
of the corresponding oligonucleotides probes immobilized
in the gel. This approach was evaluated by synthesizing
a microchip with selected probes immobilized at several

30   different concentrations, up to 6 times higher than that
used in the experiments previously described. This was
accomplished by multiple applications of the standard
loading solution (100 pmol/μl probe) to each gel element.
Comparable hybridization of Nso1225 (ammonia oxidizer)

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and Nsv443 (nitrosovibrio-like) was achieved following three applications of the Nso1225 probe (FIG. 5). Similarly, two applications of Bac338 (bacteria) and five of Uni1390 (all life) resulted in hybridization

5   comparable to Nsv443.

Strains used. *Escherichia coli*, *Desulfovibrio vulgaris* strain PT2, *Nitrosovibrio tenuis* strain NV12, *Nitrosomonas europaea* strain ATCC 19718, and *Nitrosomonas* strain C-56 were used as sources of nucleic acid for

10   these experiments.

RNA preparation. Total cellular RNA was isolated by phenol/chloroform extraction. For some of the samples, a ribosome enrichment was performed before RNA extraction. Forty ml of log phase growth *E. coli* or

15   *D. vulgaris* strain PT2 was centrifuged at 3500 g for 10 min. and resuspended in 4 ml of 4°C ribosome buffer. Ribosome enrichment buffer consisted of 20 mM MgCl2, 50 mM KCl, 50 mM Tris at pH 7.5, and 5 mM β-mercaptoethanol in diethyl pyrocarbonate treated double-distilled water.

20   The cell suspension was divided between 4 screwtop microfuge tubes and 0.5 g of 0.1 mm ZrO2 beads were added. The cell suspensions were disrupted for 2 min., put on ice for 5 min., and disrupted again for 2 min. The cell suspensions were centrifuged at 14000 g for 10

25   minutes. The supernatant, which contained the ribosomes, was recovered and transferred to ultracentrifuge tubes. Ribosomes were pelleted by ultracentrifugation in ribosome buffer at 55,000 rpm (201,000 g average) for 50 minutes in a Beckman Optima Series TL swinging bucket

30   rotor (Beckman, Fullerton, CA), for a Svedberg sedimentation factor of 70S. After centrifugation, the supernatant was discarded and the RNA was recovered from the pelleted ribosomes by extraction with pH 5.1 phenol/ chloroform. Quality and quantity of extracted RNA was

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evaluated by polyacrylamide gel electrophoresis and
ethidium bromide staining.

   **Cloning of 16S rDNA and *in vitro* production of
RNA transcripts.** DNA was extracted from *E. coli,*
5 *Desulfovibrio vulgaris* PT2, *Nitrosovibrio tenuis* NV12,
Nitrosomonas europaea 19718, and *Nitrosomonas* strain C-56
cell pastes using a guanidine/diatom method. Near-
complete 16S rDNA genes (ca. 1500 base pairs) were
recovered from each by PCR amplification using S-D-Bact-
10 0011-a-S-17 (GTTTGATCCTGGCTCAG (SEQ ID:16)) and S-D-Bact-
1492-a-A- 21 (ACGGYTACCTTGTTACGACTT (SEQ ID:17)) as
primers and a premixed PCR amplification buffer
(Pharmacia Biotech Inc. Piscataway, NJ), consisting of
0.2 mM Mg++, 2.5 mM each dATP, dCTP, dGTP, dTTP, 0.2 mM
15 of each amplification primer, and 2.5 units of Taq DNA
polymerase (Pharmacia). Temperature cycling was done in
an Idaho Technology thermocycler (Idaho Falls, ID) using
30 cycles of 15 sec at 94°C, 20 sec at 50°C, and 30 sec at
72°C. The PCR-products were cloned in a pCR plasmid
20 (Invitrogen, San Diego, CA) according to manufacturers
instructions. Plasmids were isolated using the Wizard
kit (Promega, Madison, WI) and used for *in vitro* tran-
scription of the cloned SSU rRNA genes.

   DNA oligonucleotide probes. All probes were
25 complementary to the SSU rRNAs and previously character-
ized using a membrane hybridization format. Five probes
hybridize to different groups of ammonia-oxidizing
bacteria within the beta-subdivision of the
Proteobacteria. S-G-Nso-190-b-A-19 (Nso190) and S-G-Nso-
30 1225-a-A-20 (Nso1225) encompass all sequenced ammonia-
oxidizers of the beta-subclass of *Proteobacteria*, probe
S-G-Nsm-156-a-A-19 (Nsm156) identifies members of the
genus *Nitrosomonas* (also including *Nitrosococcus*

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mobilis), probe S-G-Nsv-443-a-A-20 (Nsv443) is specific

for the *Nitrosovibrio/Nitrosolobus/Nitrosospira* group,

and probe S-G-Nsm-653-a-A-18 (NEU23) is specific for the

halotolerant members of *Nitrosomonas*. Probes for members

5   of genus *Nitrobacter* (nitrite oxidizing) were S-G-Nit-

1000-b-A-15 (Nb1000) and S-G-Nit-1035-a-A-18 (NIT3).

Other probes used were S-D-Bact-0338-a-A-18 (Bac338)

which hybridizes to members of the bacterial domain; S-D-

NBac-0338-a-S-18 (NonBac338), complementary to the

10   antisense strand of the Bac338, and S-*-Univ-1390-a-A-18

(Uni1390) complementary to the SSU RNA of nearly all

characterized living organisms, with the exception of

some protists.

        RNA and DNA labeling and fragmentation. Single

15   stranded DNA was prepared by asymmetric PCR according to

Ausubel *et al.* (1994) using a 100 times excess of the

forward primer. Briefly, DNA was partially depurinated

in 80% formic acid for 30 min. at 20°C, then incubated in

0.5 M ethylenediamine hydrochloride (pH 7.4) for 3 hr at

20   37°C, followed by 30 min. at 37°C in the presence of 0.1 M

NaBH4. Fluorescein isothiocyanate was incorporated into

fragmented DNA by incubation in absolute DMSO at room

temperature for 1 hr.

        RNA was fragmented by base hydrolysis and

25   dephosphorylated with bovine phosphatase. Fragmented RNA

was oxidized by NaIO4 and labeled either by

ethylenediamine mediated coupling of 6-carboxyfluorescein

(FAM) succinamide or by direct incorporation of

tetramethylrhodamine-hydrazide (TMR).

30   **Microchip fabrication.** A matrix of glass-

immobilized gel elements measuring 60 x 60 x 20 or 100 x

100 x 20 μm each and spaced apart by 120 or 200 μm

respectively was prepared. The polyacrylamide gel was

activated by substitution of some amide groups with
hydrazide groups by hydrazine-hydrate treatment.
Oligonucleotides were activated by oxidizing 3'-terminal
3-methyluridine using NaIO4 to produce dialdehyde groups

5   for coupling with hydrazide groups of the gel and coupled
to each micromatrix element by applying 0.5-1 nl of the
activated oligonucleotide solution (100 pmol/μl) using a
specially devised robot.

Hybridization and image analysis.   Probe

10   binding was quantified by measuring the fluorescence
conferred by the binding of fluorescently labeled DNA or
RNA (tetramethyl rhodamine or fluorescein) to the
individual gel elements. Hybridization and washing was
controlled and monitored using a Peltier thermotable

15   (with a working range of -5.0°C to +60.0°C) mounted on the
stage of a custom-made epifluorescent microscope. The
microchip was hybridized at 5°C, either overnight or for
6 hr, in 2-5 μl of the hybridization buffer [33%
formamide, 0.9 M NaCl, 1 mM EDTA, 1% Tween-20, and 50 mM

20   sodium phosphate (pH 7.0)] at a concentration of DNA and
RNA between 0.2-2 pmol/μl. The hybridization mixture was
replaced with 5-10 μl hybridization buffer without
formamide immediately prior to microscopic observation.
Exposures were in the range of 0.1-10 sec depending on

25   the signal intensity, but were typically around 1 sec.
Fluorescence was monitored either at room temperature or
using a range of temperatures between 5 - 60°C.

Conditions for the coupling of micromolecules
to the acrylamide gel were devised to rule out the

30   possibility of liquid evaporation during immobilization
and to ensure that covalent bonding of oligonucleotides
with the gel matrix proceeds to completion. After the
microvolumes of the oligonucleotide solutions have been
applied to all cells of the matrix, the micromatrix gel

elements were swelled by condensing water from the
ambient air. Then the micromatrix surface was covered
with a thin layer of an inert nonluminescent oil, and
chemical coupling of the activated oligonucleotides to
5   the activated polyacrylamide was carried out to
completion.

    Example 6: **Use of Microchip Biosensors As**

Diagnostic Assays

            The microchip technology was successfully
10   tested for identification of single base changes in
genomic DNA and RNA for reliable diagnosis of human
genetic diseases. A customized microchip contained
oligonucleotides specific to β-thalassemia normal and
abnormal β-globin genes. The hybridization with PCR-
15   amplified DNA or RNA samples derived from genomic DNA of
subjects allowed unambiguous identification of a mutation
in a sample to be tested. Reliability of the identifica-
tion was enhanced by using simultaneous hybridization
with two samples of a normal and mutated RNA stained with
20   different fluorescence dyes and monitoring the
hybridization at different wavelengths; by simultaneously
measuring the melting curve for duplexes formed on a
microchip, and by using a proper set of several
oligonucleotides complementary to the mutated site of the
25   DNA.

            A number of the most commonly occurring
β-thalassemia mutations with β-globin gene were used in
diagnostic assays with oligonucleotide microchip
biosensors. These mutations were splice-site mutations
30   for the 1st, 2nd, 5th, and 6th nucleotides in the first
intron (IVS I) of the β-globin gene: IVS I/1 G/A (G/A =
substitution of G for A), IVS I/2 T/C, IVS I/5 G/T, IVS
I/5 G/C, IVS I/6 T/C, and G/A substitution in the 26th
codon (GAG) of the first exon (FIG. 6), (also known as

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abnormal hemoglobin E) (see Diaz-Chico et al., 1988 for
terminology).

A microchip with 100×100×20 μm gel elements
(Yershov et al., 1996) contained immobilized
decadeoxyribonucleotides, that is, 10-mers that
correspond to normal and mutant β-thalassemia alleles.
These 10-mers discriminated mismatches less reliably than
8-mers, but were hybridized more efficiently than 8-mers.
10-mers were, therefore, preferred for this assay.
Table 3 shows the sequences of the allele-specific
oligonucleotides immobilized on the microchips. It was
expected that mismatches within the duplexes would have a
much higher destabilization effect than mismatches at the
terminal positions (Khrapko et al., 1991); therefore the
mutated bases were placed inside of the immobilized
oligonucleotides.

Single- and double-stranded PCR-amplified β-
globin DNA fragments of different lengths and collected
after a random fragmentation were tested in assays for
identification of some of these mutations. However, the
hybridization of RNA is preferred over DNA hybridization.
RNA fragments were derived from PCR-amplified genomic DNA
by transcription with T7 RNA polymerase (Lipshutz et al.,
1995). About 100 copies of unlabeled or fluorescently
labeled RNA transcripts are synthesized per DNA molecule,
providing a convenient way to prepare a sufficient amount
of the hybridization probes. RNA is fragmented and one
fluorescent dye molecule is introduced per fragment.

Table 3 shows the sequences of the microchip
allele specific 10-mers. The oligonucleotides of
microchip I are complementary to the coding strand of DNA
of the β-globin gene of patients with β-thalassemia
single-base mutations (G/A - substitution of A for G) in
the 1st, 2nd, 5th, or 6th nucleotides of the first intron

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(IVS I/1, 2, 5, 6) of the β=globin gene and in the codon
#-26 (CD-26) of the first exon. Oligonucleotides 1-16 of
microchip II correspond to the normal and IVS I/2 G/T
allele. The mutated and corresponding normal bases are
placed from the 2nd to the 9th positions of the 10-mers
from their 3'-end. The mutated bases are shown in
lowercase bold letters and corresponding oligonucleotide
bases in the normal allele are underscored. The
oligonucleotide synthesis and the microchip manufacturing
were described by Yershov et al. (1996).

    Microchip I was successively hybridized with
RNA 75 and 133 nt long without fragmentation or after
fragmentation (133fr, Table 5, probes 3a and 4a) and with
6 synthetic 19-mer oligodeoxyribonucleotides corre-
sponding to β-thalassemia mutations. The RNA and 19-mers
were labeled with TMR except for RNA probes 2a, 2b, and
6b, which were labeled with fluorescein (Fl). The
melting curves (FIG. 1A-B, FIG. 2) were measured
simultaneously for all microchip oligonucleotides at each
hybridization. These curves provided values of
hybridization intensities at the discrimination
temperature, Td. R is the ratio of the hybridization
signal of a mismatched duplex (Im) to the signal of the
perfect duplex (Ip) estimated at Td in parallel for all
microchip oligonucleotides. $R = Im/Ip$. d_{19}-synthetic 19-
deoxymers were complementary to allele specific 10-mers
immobilized on the microchips.

    Table 4 shows the effect of the position of the
allelic base within 10-mers on mutation detection.
Microchip II contains two sets of 10-mers corresponding
to the normal and IVS I/2 T/G alleles. The microchip was
hybridized with the TMR-labeled normal allele 19-mer and
to an RNA 75 nt long. $T_{0.1}$ is the temperature at which
the hybridization signals for a microchip duplex drops to

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1/10 of its initial value at 0°C. $-\Delta T_{0.1} = T_{0.1}$ (a perfect duplex) minus $T_{0.1}$ (the corresponding mismatched duplex.)

Fluorescently labeled RNA probes were prepared from a fragment of the β-globin gene from the first exon (Lawn *et al.*, 1980). PCR amplification of a 1.76-kb fragment of the human β-globin gene mapped from nucleotides -47 to +1714 (Lawn *et al.*, 1980) was carried out with 1 µg genomic of DNA (Poncz *et al.*, 1982) and 50 pmol each of the forward primer: 5'-GGAGCCAGGGCTGGGCATAAAAGT-3' (SEQ ID:18)) (-47->-23) and the reverse primer 5'-ATTTTCCCAAGGTTTGAACTAGCTC-3' (SEQ ID:19)(+1689->+1714). (FIG. 7) The amplification was carried out in a DNA thermal cycler (GeneAmmp PCR System 2400, Perkin Elmer Corporation) in 100 µl of a buffer containing 200 mM each of dATP, dCTP, dGTP, dTTP, 2.5 mM $MgCl_2$, 2 units of Taq DNA polymerase (BioMaster, Russia), 50 mM KCl, 10 mM Tris-Hcl, pH 9.0, and 0.1% Triton X-100. The reaction conditions were 30 cycles, with 45 sec at 95°C, 90 sec at 66°C, and 120 sec at 72°C. PCR product was purified from 2% low gel/ melting temperature agarose gel (NuSieve agarose, FMC). The 159 bp and 102-bp DNA fragments were amplified with 10 ng of the 1.75 kb DNA with three nested primers, two containing T7 promoter sequence and a common reverse primer. The nested primers were T7-V2L-45.

(5'-GGAATTCCTAATACGACTCACTATAGGGA
CACCATGGTGCACCTGACTCC-3' (SEQ ID:1) -44->+66); T7-V2L-103
(5'-GGAATTCCTAATACGACTCACTATAGGGAGGTGAACGTGGATGAAGTTGG-3'
(SEQ ID:2); +102->-123); and 5'-TCTCCTTAAACCTGTCTTGTAACC-3' (SEQ ID:3) (common reverse;153->+176). The amplification was carried out in 25 cycles (15 sec at 95°C, 30 sec at 62°C, and 30 sec at 72°C). PCR products were purified by QIAGEN QIAquick PCR Purification Kit.

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The PCR-amplified 159 or 102 bp DNA (4-5 μg) containing
T7 promoter was transcribed with 400 units of T7 RNA
polymerase (Promega) to produce 133 and 75 nt long RNA in
80 μl of buffer containing 300 mM HEPES, pH 7.6, 30 mM

5   MgCl$_2$, 16 μg of BSA, 40 mM DTT, 30 units of Rnasin
(Promega) and 4 mM each of ATP, CTP, GTP, and UTP for 3 h
at 38°C. Deproteinization of the reaction mixture was
carried out in 20 mM EGTA, pH 8.0, 2% SDS, and Proteinase
K (10 mg/ml) for 15 min at 37°C. The mixture was

10   extracted first with equal volumes of phenol and then
with equal volumes of chloroform, precipitated twice by
one volume of isopropyl alcohol, from 0.5 M LiClO$_4$ and
dissolved on a Bio-Spin P6 column (BioRad).

        Fragmentation of 10-100 μg of RNA to an average

15   length of 20- to 40-mers was carried out in 50 μl of 0.1
M KOH for 30 min. at 40°C. Then 5 μl of 1M HEPES, pH
7.6, and 15 μl of 1% HCO$_4$ were added at 4°C. The pellet
of potassium perchlorate was removed by centrifugation
and RNA was precipitated by 10 volumes of 2% LiClO$_4$ in

20   acetone. The RNA was washed twice with acetone and dried
for 20-30 min. at room temperature. The fragmented RNA
was dephosphorylated in 50 μl of 20 mM Tris-HCl, ph 8.0,
1 mM MgCl$_2$, 1 mM ZnCl$_2$, 10 units of Rnasin, 5-7 units of
calf intestine phosphatase (CIP) for 1 hour at 37°C. RNA

25   deproteinization and purification was carried out as
described herein.

        For chemical fluorescence labeling of RNA the
3'-terminal dephosphorylated nucleoside was oxidized in
20 μl of 10 mM sodium periodate for 20 min. at room

30   temperature. RNA was precipitated with acetone. A 10
molar excess of 10 mM TMR-hydrazine in 10% acetonitrile
was added to oxidized RNA fragments in 20 μl of 20 mM
sodium acetate at pH 4.0.

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The reaction mixture was incubated 30-40 min at 37°C, and the hydrazide bond between the RNA and dye was stabilized by reduction with freshly prepared 1.5 µl of 0.2 M NacNBH₃ and incubated for 30 min. at room

5   temperature. Then the mixture was extracted four times with water saturated n-butanol and precipitated with acetone. Alternatively, RNA was labeled by incorporation of fluorescein-UTP during the transcription with Ambion MEGAshortscript kit according to the manual.

10   The hybridization of fluorescently labeled RNA (1 pmol/μl) with the microchips was carried out at 0°C for 18 h. In many cases, the intensities of the hybridization signals at 0°C were similar for perfect and mismatched duplexes. The perfect and mismatched duplexes

15   as well as the duplexes having various GC and AT contents displayed different stabilities and therefore were tested at different temperatures.

Table 4 summarizes the results of hybridization of the diagnostic microchips with 1) RNA probes derived

20   from a number of homozygous and heterozygous β-thalassemia patients; and 2) with corresponding 19-mers. The table shows the Td for perfect duplexes formed on each microchip oligonucleotide. The relative intensities, R, of the hybridization signals for a

25   different microchip oligonucleotides in Table 3 are normalized to the signals for a perfect duplex at the Td (estimated as 1.0). In most cases the ratios for mismatched duplexes are less than 0.1 and close to 0. These values are low enough to allow unambiguous

30   identification of the homozygous and heterozygous mutations in patients at the Td (when the hybridization signals from only perfect duplexes are observed). The hybridization of homozygote RNA (Table 5, probes 1a, 2a, 2b, and 3a) with the microchip shows the distinctive

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formation of a perfect duplex only with one immobilized
oligonucleotide and mismatched duplexes with all others.
Two perfect duplexes were unambiguously identified upon
hybridization with a heterozygote RNA (Table 5,
5   probe 4a).

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Table 3.   The sequence of the microchip
allele specific 10-mers.

	#	Allele	Position of mutated base	Sequence	Location
M	1	IVS (N)	-	5'-A TAC CAA CCT-gel (SEQ ID:20)	+141
	2	IVS I/1 G/A	8	5'-A TAC CAA tCT-gel (SEQ ID:21)	+141
I	3	IVS I/1 G/T	8	5'-A TAC CAA aCT-gel (SEQ ID:22)	+141
C	4	IVS I/2 T/A	7	5'-A TAC Cat CCT-gel (SEQ ID:23)	+141
R	5	IVS I/2 T/C	7	5'-A TAC Cag CCT-gel (SEQ ID:24)	+141
O	6	IVS I/2 T/G	7	5'-A TAC Cac CCT-gel (SEQ ID:25)	+141
C	7	IVS I/5 G/A	4	5'-A TAt CAA CCT-gel (SEQ ID:26)	+141
H	8	IVS I/5 G/C	4	5'-A TAg CAA CCT-gel (SEQ ID:27)	+141
I	9	IVS I/5 G/T	4	5'-A TAa CAA CCT-gel (SEQ ID:28)	+141
P	10	IVS I/6 T/C	3	5'-A TgC CAA CCT-gel (SEQ ID:29)	+141
	11	CD 26 (N)	-	5'-G GCC TCA CCA-gel (SEQ ID:30)	+125
I	12	CD 26 G/A	6	5'-G GCC TtA CCA-gel (SEQ ID:31)	+125
	1	IVS (N)	9	5'-TGA TAC CAA C-gel (SEQ ID:32)	+143
	2	IVS I/2 T/G	9	5'-TGA TAC CAc C-gel (SEQ ID:33)	+143
M	3	IVS (N)	8	5'-GA TAC CAA CC-gel (SEQ ID:34)	+142
I	4	IVS I/2 T/G	8	5'-GA TAC CAc CC-gel (SEQ ID:35)	+142
C	5	IVS (N)	7	5'-A TAC CAA CCT-gel (SEQ ID:36)	+141
R	6	IVS I/2 T/G	7	5'-A TAC Cac CCT-gel (SEQ ID:37)	+141
O	7	IVS (N)	6	5'-TAC CAA CCT G-gel (SEQ ID:38)	+140
C	8	IVS I/2 T/G	6	5'-TAC CAc CCT G-gel (SEQ ID:39)	+140
H	9	IVS (N)	5	5'-AC CAA CCT GC-gel (SEQ ID:40)	+139
I	10	IVS I/2 T/G	5	5'-AC CAc CCT GC-gel (SEQ ID:41)	+139
P	11	IVS (N)	4	5'-C CAA CCT GCC-gel (SEQ ID:42)	+138
	12	IVS I/2 T/G	4	5'-C CAc CCT GCC-gel (SEQ ID:43)	+138
I	13	IVS (N)	3	5'-CAA CCT GCCC-gel (SEQ ID:44)	+137
I	14	IVS I/2 T/G	3	5'-CAc CCT GCC C-gel (SEQ ID:45)	+137
	15	IVS (N)	2	5'-AA CCT GCC CA-gel (SEQ ID:46)	+136
	16	IVS I/2 T/G	2	5'-Ac CCT GCC CA-gel (SEQ ID:47)	+136

In Table 3, mutated oligonucleotide bases are
shown in lower case and bold, and corresponding oligo-
5   nucleotide bases in the corresponding normal alleles are
underlined.

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TABLE 4
The effect of the position of the allele base
within 10-mers on mutation detection.

5

Position allele	19-mer			RNA		
	$T_{0.1}$ of perfect	$T_{0.1}$ of (G-A)	$\Delta T_{0.1}$	$T_{0.1}$ of perfect	$T_{0.1}$ of (G-A)	$\Delta T_{0.1}$
9	40	32	8	35	37	-2
8	47	32	15	49	38	11
7	42	30	12	44	41	3
6	47	28	19	49	41	8
5	52	38	14	50	42	8
4	54	39	15	54	44	10
3	55	46	9	59	54	5
2	52	46	6	58	53	5

TABLE 5
Identification of β-thalassemia
mutations by hybridization with the microchip.

#	Allele	Size (nt)	Immobilized 10-mer oligonucleotide											
			IVS (N)	I/1 G/A	I/1 G/T	I/2 T/A	I/2 T/C	I/2 T/G	I/5 G/A	I/5 G/G	I/5 G/T	I/6 T/C	CD26 (N)	CD26 G/A
			R at Td=											
			42°C	39°C	38.5°C	42°C	48°C	45.5°C	37°C	44.5°C	40°C	50°C	54.5°C	49°C
1 a	IVS (N)	75	1.0	0.04	0	0.20	0.05	0.07	0	0	0	0.04	1.0	-
b	IVS (N)	19ª	1.0	0.09	0.07	0.02	0.03	0.01	0.03	0.03	0.07	ND	0	0
2 a	IVS I/2 T/A	F1 75	0.15	0	0	1.0	0.12	0.08	0	0	0	0	1.00	-
b	IVS I/2 T/A	F1 133	0.03	0	0	1.0	0	0.30	0	0	0	0	1.00	0.19
c	IVS I/2 T/A	19ª	0.01	0	0	1.0	0.07	0.03	0	0	0	0	0.01	0
3 a	IVS I/1 G/A	133fr	0.03	1.0	0	0.01	0	0.02	0	0	0	0	1.00	-
b	IVS I/1 G/A	19c	0.01	1.0	0.01	0.01	0	0	0.01	0	0	0	0	0
4 a	IVS I/1 G/A & IVS I/6 T/C	133fr	0.2	0.85	0	0.2	0	0.05	0	0	0	1.00	1.00	-
b	IVS I/1 G/A	19c	0.01	1.0	0.01	0.01	0	0	0.01	0	0	0	0	0
c	IVS I/6 T/C	19ª	0.1	0	0	0	0	0	0	0	0	1.0	0	0

(Table 5 continued on next page)

Hybridized Probe			Immobilized 10-mer oligonucleotide											
			IVS (N)	I/1 G/A	I/1 G/T	I/2 T/A	I/2 T/C	I/2 T/G	I/5 G/A	I/5 G/G	I/5 G/T	I/6 T/C	CD26 (N)	CD26 G/A
#	Allele	Size (nt)	R at Td=											
			42°C	39°C	38.5°C	42°C	48°C	45.5°C	37°C	44.5°C	40°C	50°C	54.5°C	49°C
5 a	IVS I/5 G/T	19*	0	0	0	0	0	0	0.03	0.02	1.00	0	0	0
5 b	CD26 (N)	19c	0	0	0	0	0	0	0	0	0	0	1.00	0.03
5 c	CD26 G/A	19*	0.03	0	0	0	0	0	0	0	0	0	0.04	1.00
6 a	IVS (N)	75	1.00	0.04	0	0.20	0.05	0.07	0	0	0	0.04	1.00	-
6 b	IVS I/2 T/A	F1 133	0.03	0	0	1.00	0	0.30	0	0	0	0	1.00	-

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The noticeable exceptions are oligonucleotides corresponding to IVS ½ T/A and IVS ½ T/G mutations that show strong mismatched signals upon hybridization with non-corresponding samples of IVS (N) and IVS ½ T/A RNA's,

5   respectively (Table 5, 1a, 2b, 4a, 6a and 6b). The relative intensities of these mismatched signals can be significantly decreased by choosing the proper oligonucleotides for immobilization. It appears that the diagnostic assays can be carried out with RNAs 75

10   nucleotides (nt) long (Table 5, probes 1a, and 6a), and 133 nt long (probes 2a and 6b), as well as with 133 nt long RNA fragmented to pieces 20-40 nt long (probes 3a and 4a). However, the intensities of the hybridization signals after fragmentation are increased by about 5

15   times and the time of hybridization is decreased from several hours to a tens of minutes.

The longer RNA probes diffuse more slowly into the gel and can form stable secondary structures or aggregates. These factors interfere with their

20   hybridization with rather short immobilized oligonucleotides. Thus, the fragmentation seems to be an essential step in sample preparation, since it enhances and speeds the hybridization.

In addition to the measuring of the melting

25   curves, the reliability of identification of mutations and base changes can be enhanced by the use of a multicolor fluorescence microscope (Yershov et al., 1996). For this purpose, the tested RNA is marked by one fluorescence label and is hybridized with a microchip in

30   the presence of a normal allele sample labeled with a different dye. The pattern and the ratio of hybridization measured with the two dyes will be similar for all microchip oligonucleotides except for those that correspond to different allele bases, i.e., mutations.

35   Table 4 shows the results of such an experiment. The patterns of hybridization detected at two wavelengths are

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very similar.

As shown in Table 3, the immobilized 10-mers
matching the mutations IVS I-2 T/G, IVS I-2 T/C, and IVS
I-2 T/A are hybridized rather strongly with some RNA
5   probes that correspond to other alleles. Different
structural factors in RNA could cause this hybridization.
The effect of these factors can be minimized by placing a
variable IVS I-2 base into different positions of the
10-mers. The results of such experiments are shown in
10   Table 4. Microchip II was successively hybridized with
fragmented 75-nt-long RNA or with a synthetic DNA 19-mer,
both corresponding to the normal allele. Microchip II
contained two similar sets of eight overlapped
immobilized 10-mers that are complementary either to a
15   normal allele or to IVS I-2 T/G allele. The allele
specific bases A for the first set and C for the second
set are located in these 10-mers in all internal
positions from the 2nd to the 9th. These bases form
perfect A-T or mismatched A-G base pairs, respectively.
20   The stability of the perfect and mismatched duplexes
formed on the microchip is determined as $T_{0.1}$ the
temperature at which the initial hybridization signal of
the duplex is decreased to one-tenth of the original
intensity. $\Delta T_{0.1}$ corresponds to the difference in $T_{0.1}$
25   between the perfect and similar mismatched duplexes. A
better discrimination of the perfect and mismatched
duplexes is reflected in higher values of $\Delta T_{0.1}$. The
discrimination efficiency (ΔT) was lower for hybridized
RNAs than for the 19-mers. The discrimination was
30   surprisingly low, $\Delta T = -2°$ and $3°C$, when the allelic
bases were placed at the 9th or 7th position, respectively,
of the immobilized oligonucleotides. It appears that
secondary structures and the presence of similar
sequences in other regions of the RNA causes this
35   lowering. These effects can be partly predicted from the

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sequence of the region that is searched for mutations. However, it is impossible to reach a high discrimination (ΔT =8-11°C) when allele bases are placed in other positions, for example the 8, 6, 5, or 4 positions.

The hybridization of RNA transcripts of PCR-amplified DNA with oligonucleotide microchips allows the reliable identification of base changes and discrimination of homozygous and heterozygous β-thalassemia mutations in the genomic DNA of patients.

RNA transcribed from PCR-amplified DNA provides an easier method for preparing a sufficient amount of labeled, single-stranded samples than the use of DNA prepared by PCR amplification. RNA can be fragmented and one fluorescent dye molecule can be introduced per fragment.

Example 7: **Use of a Customized Microchip Biosensor to Detect Gene Expression**

Gene expression is one of the central themes in modern molecular biology. DNA from well studied genetical sources has already been systematically sequenced. For these sequences hybridization procedures are successfully used to estimate a level of differential gene expression. The results of this estimation are useful for understanding fundamental mechanisms of development biology, embryology and treatment of genetic and infectious diseases.

To determine whether oligonucleotide microchips are useful to identify gene expression, microchip biosensor hybridization was carried out with ssDNA fragments isolated from six different genes:

- 205 b fragment from glyceraldehyde 3-phosphate dehydrogenase (G3PDH);

- 281 b fragment from human transferrin receptor (HTR);

- 224 b fragment from human β_2-microglobulin (B2M);

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        - 545 b fragment from human interleukin-1 receptor
(IL1R);

        - 188 b fragment from human NF-kB (p50);

        - 224 b fragment from human interferon γ receptor
5   (IGR).

        A customized microchip, containing immobilized
60 b oligonucleotides, having at the 3'-terminal position
3-methyluridine residues, corresponding to five house-
keeping genes (G3PDH, HTR, B2M, IL1R and NF-kb(p50))
10   (CLONTECH catalog 94/95 "Tools for the Molecular
Biologist", pp. 90-93) were produced for hybridization
experiments with complementary ssDNA fragments. Each
oligonucleotide was applied at two positions on the
microchip in a 1:10 ratio of amount (0.3 pmol:0.03 pmol
15   each). ssDNA fragments complementary to immobilized
oligonucleotides were synthesized by asymmetric PCR
amplification (using only one primer) with fluorescently
labeled nucleotide triphosphates (*FUORscript T7*,
Fluorescein-Labeling In Vitro Transcription Kit).
20   Moreover, the PCR-primer bore a biotin tag that was
utilized for following isolation of synthesized ssDNA
fragments with avidin carried on a column (Sambrook
et al. "Molecular Cloning" 2d edit., p. 12.14). FIG. 8
demonstrates hybridization on the microchip. Intensity
25   of fluorescence in each spot depends on the amount of
immobilized oligonucleotide and on the length of the DNA
fragment in the spot. For hybridization, 10 μl of Buffer
A (50% formamide, 10% dextran sulfate, 1% SDS, 50 mM
sodium phosphate at pH 7.4, 750 mM sodium chloride, 5 mM
30   sodium EDTA) containing ssDNA with a concentration of 0.5
pmol/μl (approximately 0.05 μg/μl) was incubated for
about 6 - 12 h at room temperature, washed briefly with
H_2O and analyzed with a fluorescent microscope. Before
rehybridization the microchip was treated in Buffer B
35   (50% formamide, 1% Tween 20) for 30 min. at 50°C to

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completely remove hybridized ssDNA.

These results indicate that concentration of
fluorescently labeled ssDNA may be decreased up to 100
fold. Hybridization with individual ssDNA fragments
5   indicates high specificity of studied oligonucleotides.
There was no cross-hybridization detected between
different tested DNAs and immobilized oligonucleotides.
None of the oligonucleotides demonstrated a signal when
hybridized with non-specific DNA (e.g. probe IGR). This
10   differentiates "expression" of non-expressed genes from
expression of housekeeping genes. Genes that are not
expressed in a particular cell or tissue, may actually be
picked up in conventional screening procedures as having
a low expression, while other genes being expressed in
15   all cells (housekeeping genes) will also be picked up as
having low to moderate expression. The housekeeping
genes are actually being expressed. In this example a
difference in signal is detectable so that low level
expression could be unambiguously distinguished from low
20   level background.

The procedure detects expression of genes of
high and middle expression level. To determine low level
gene expression selective RT-PCR amplification is
preferred.

25   **Example 8**: **Use of a Customized Microchip Biosensor
to Detect HLA Polymorphisms**

A difficult problem of genotype recognition
arises in studying different haplotypes (alleles) of
genes encoding Human Leucocyte Antigens (HLA) in regions
30   of histocompatibility genes. The HLA locus (class I and
class II genes) is responsible for histocompatability of
tissue transplantation. The need for allele
identification is encountered also in various medical and
biological tasks involving HLA class II genes. There are
35   many clinical data showing strong association between HLA
genotype and suspectibility to some disorders, for

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example some alleles DQA1/DQB1 are clearly related to
IDDM (Insulin - Dependent Diabetes Mellitus), malaria,
autoimmune diseases, such as rheumatoid arthritis and
pemphigus vulgaris - a skin disease which causes severe

5   blistering. The high level of polymorphism of HLA has
been shown to be useful for identification of individuals
determining the group of risk for some diseases. HLA
typing is particularly crucial for matching donors for
transplants. It is also proposed for infertility work-

10   ups.

In this aspect, the present invention provides
a method which allows an array of immobilized 8-12 bp-
long oligonucleotides to form an oligonucleotide
microchip thereby facilitating identification of HLA DQA1

15   allies.

An algorithm has been designed and special
computer programs have been constructed which allow the
analysis of the nucleotide sequences of all alleles of
various HLA subloci. Forming an optimized set of

20   oligonucleotides provides high reliability of detection
of homo- and heterozygotes for the HLA alleles.

A customized microchip, containing an array of
eighteen PAA - gel immobilized (1pmol of each) short
oligonucleotides has been produced for hybridization with

25   fluorescently labelled complementary HLA DQA1 DNA or RNA
probe for allele identification. 18 decamers were loaded
on the chip in the following order, from left to right:

TABLE 6				
first (upper) row	1	2	3	4	g^4-control oligo
second:	5	6	7	8	g^3-control oligo
third:	9	10	11	12	13
4-th:	14	15	16	17	18

30

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The sequence of the oligos used was as shown in
FIG. 9.

The oligonucleotides immobilized on the
microchip are complementary to the sense strand of
5   different alleles of DQA1 DNA and some control oligos. A
microchip with 20 oligonucleotides was manufactured for
partial identification of 15 different alleles in the HLA
DQA1 region. PCR was used to prepare 229 bp (starting
from codon 12 to codon 87) DNA fragments of the
10   polymorphic second exon of the DQA1 gene from human
genomic DNA. Nested primers were used 2DQAAMP-A:5'-
a t ggt gta aac ttg tac cag t; and 2DQAAMP-
B:5'tt ggt agc agc ggt aga gtt g. Nested PCR primers
were: T7-2DQAAMP-A and primer B. The first primer
15   containing the promoter for T7 RNA polymerase and PCR
product were used for *in vitro* transcription. RNA probes
were identical to the coding DNA strand. RNA was
fragmented, labelled with fluorescein and used for
hybridization with the microchip. Hybridization
20   conditions were as follows: overnight incubation at 5°C
in 1M NaCl, 1mM EDTA, 5 mM Na-phosphate, pH7.0, 1% Tween
20. The temperature was then increased stepwise at 10°C
intervals, and fluorescence measurements were taken at
each step.
25   FIG. 10 shows the hybridization results and
presents schematically the HLA DQA1 - chip for allele
identification. In FIG. 10 three diagonally placed
oligos (11-0101/0104 allele specific; 6-specific for
0101,01021,01022,0103,00104; 17-correspond to all
30   alleles, except 0502) gave a positive hybridization
signal, and are observed as three diagonally placed
bright fluorescence spots. The probes were identified as
the 0101 or 0104 allele (both alleles are identical in
the second exon). All other oligos yielded much weaker
35   fluorescence signals compared with those described above,

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because none of them contain sequences complementary to
alleles 0101 and 0104. On the other hand any allele
different from 0101 or 0104, reveals another set of
hybridization signals.

5   The brightest fluorescent squares on the chip were:
Oligo# 4 which is 03011 or 0302 specific; oligo# 8 is Taq
polymerase-specific artifacts; oligo# 18-belong to
alleles 0101-05011; oligo 11-0101,0104 allele specific;
17 - corresponds to all alleles, except 0502; g4 is a

10   fluorescent control oligo; #13 - mismatch to #18. All
other chip elements showed significantly less intensive
fluorescence. The genotype identified by these probes
has a 0101/0104-0302/03011 heterozygote.

Example 9: Use of a Customized Microchip Biosensor

15 **to Detect the Lyme Disease Spirochetes**

Bacteria belonging to the species *Barretia
burgdorferi* and related species of tick-borne spirochetes
are capable of causing human and veterinary disease.
Nucleic acid probes are available to detect bacteria

20   causing Lyme disease. These bacteria cannot be
identified by standard microbiological methods, although
immunological tests are available.

Using the methods of the present invention,
oligonucleotides are prepared according to Weisburg

25   (1995) and added to a micromatrix designed for use in
detecting Lyme disease in a clinical sample.

Example 10: Use of a Customized Microchip Biosensor

to Detect Salmonella In Food Samples

Salmonella presence is detected most commonly

30 by preparing cultures according to standard micro-
biological laboratory procedures, and testing the
cultures for morphological and biochemical character-
istics. After about 48 hours after collection of a
sample testing begins and takes several days to complete.

35   However, RNA and DNA probes for Salmonella

testing are available. Using the methods of the present
invention, oligonucleotides are prepared according to
Lane *et al.* (1996) incorporated herein by reference and
added to a microchip designed for use in detecting
5   Salmonella in food samples by distinguishing rRNA of
Salmonella from non-Salmonella.

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WE CLAIM:

1.   A method for using a microchip to identify a
nitrogen base sequence in a nucleic acid of a sample,
said method comprising:
         a)   extracting the nucleic acid from said
sample;
         b)   providing a customized matrix of
oligonucleotides having a sequence identity on the
microchip designed to identify the nitrogen base sequence
of an oligonucleotide in the sample;
         c)   hybridizing said extracted nucleic acid on
said microchip; and
         d)   identifying the nitrogen base sequence in
said sample by comparing the sequence identity of the
microchip oligonucleotides which hybridized to the sample
nucleic acid and the sequence identity of the microchip
oligonucleotides which did not hybridize.

2.   The method of claim 1, wherein said nitrogen
base sequence is in a DNA molecule.

3.   The method of claim 1, wherein said nitrogen
base sequence is in a 16S RNA, mRNA or other RNA
molecule.

4.   The method of claim 1, wherein the customized
matrix of oligonucleotides affixed to a support is formed
by a plurality of gel elements, the number of elements is
determined by the number of oligonucleotides in the
matrix; and wherein each gel element contains one
oligonucleotide of a desired nitrogen base sequence
length and concentration; said gel elements being
separated from one another by hydrophobic glass spaces;
and wherein the gel elements have a vertical height above
the plane of the interstitial spaces of not more than

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about 30 μm.

5.   The method of claim 4, wherein said
oligonucleotide matrix comprises oligonucleotides having
the base sequences

Oligonucleotide Name and Position	Sequence (5' to 3')
Nb1000	5'-TGC GAC CGG TCA TGG-3' (SEQ ID:6)
NIT3	5'CCT GTG CTC CAT GCT CCG-3' (SEQ ID:7)
NEU23	5'-CCC CTC TGC TGC ACT CTA-3' (SEQ ID:8)
NSO190	5'-CGA TCC CCT GCT TTT CTC-3' (SEQ ID:9)
NSO1225	5'-CGC GAT TGT ATT ACG TGT GA-3' (SEQ ID:10)
NSMO156	5'-TAT TAG CAC ATC TTT CGA T-3' (SEQ ID:11)
NSV443	5'-CCG TGA CCG TTT CGT TCC-3' (SEQ ID:12)
BAC338	5'-GCT GCC TCC CGT AGG GAT-3' (SEQ ID:13)
NonBAC338	5-'ACT CCT ACG GGA GGC AGC-3' (SEQ ID:14)
UNI1390	5'GAC GGG CGG TGT GTA CAA-3' (SEQ ID:15)

6.   The method of claim 5, wherein the
oligonucleotides are arranged in the matrix

	I	II	III	IV
A	Nb1000	NIT3	NEU23	Nso190
B	Nso1225	Nsm156	Nsv443	
C	Bac338	NonBac338	Uni1390	

7.   The method of claim 1, wherein the sample is
water.

8.   The method of claim 1, wherein the sample is
blood.

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9.   The method of claim 1, wherein the sample is a food.

10.   The method of claim 1, further comprising:
        e)   adding a label to the nitrogen base sequence in said sample before bringing it in contact with the array.

11.   The method of claim 10, wherein the label is a fluorescent dye.

12.   The method of claim 10, wherein the label is a plurality of different dyes.

13.   The method of claim 1, wherein the oligonucleotides on the microchip are complementary to the beta globin gene.

14.   The method of claim 1, wherein the oligonucleotides on the microchip are complementary to a sequence specific for Salmonella.

15. A microchip matrix for the detection and classification of nitrifying bacteria wherein said matrix has the following design:

	I	II	III	IV
A	Nb1000	NIT3	NEU23	Nso190
B	Nso1225	Nsm156	Nsv443	
C	Bac338	NonBac338	Uni1390	

and wherein the base sequences are:

Oligonucleotide Name and Position	Sequence (5' to 3')
Nb1000	5'-TGC GAC CGG TCA TGG-3' (SEQ ID:6)
NIT3	5'CCT GTG CTC CAT GCT CCG-3' (SEQ ID:7)
NEU23	5'-CCC CTC TGC TGC ACT CTA-3' (SEQ ID:8)
NSO190	5'-CGA TCC CCT GCT TTT CTC-3' (SEQ ID:9)
NSO1225	5'-CGC GAT TGT ATT ACG TGT GA-3' (SEQ ID:10)
NSMO156	5'-TAT TAG CAC ATC TTT CGA T-3' (SEQ ID:11)
NSV443	5'-CCG TGA CCG TTT CGT TCC-3' (SEQ ID:12)
BAC338	5'-GCT GCC TCC CGT AGG GAT-3' (SEQ ID:13)
NonBAC338	5-'ACT CCT ACG GGA GGC AGC-3' (SEQ ID:14)
UNI1390	5'GAC GGG CGG TGT GTA CAA-3' (SEQ ID:15)

16. A diagnostic assay for the presence of a
mutation in a gene said assay comprising:

      a.   designing a customized microchip biosensor
comprising an oligonucleotide that hybridizes to the gene
having the mutation;

      b.   contacting an assay sample to the
customized microchip biosensor under conditions that
allow hybridization of the gene to the microchip; and

      c.   determining whether hybridization occurs
from which presence of the mutation in the gene is
determined.

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17. A method for detecting mismatches between a nitrogen base sequence on a microchip and a nitrogen base sequence to be tested, said method comprising:

5   (a)   simultaneously monitoring hybridization between the nitrogen base sequence on the microchip and the nitrogen base sequence to be tested at a series of temperatures;

          (b)   selecting the temperature at which maximum

10 hybridization occur; and

          (c)   determining the degree of mismatch from the selected temperature.

18. The method of claim 17, wherein the selected

15 temperature is that temperature at which the intensity of a predetermined hybridization temperature from a perfectly matched duplex is one tenth of its initial value.

20   19. A customized oligonucleotide microchip for the detection of a betaglobin, mutation, said microchip containing space oligonucleotides have the following nitrogen base sequences with identifiers:

IVS (N)	5'-TMR-CCTGGGCAGGTTGGTATCA-3'	(SEQ ID:48);
IVS I/2 T/A	5'-TMR-CCTGGGCAGGaTGGTATCA-3'	(SEQ ID:49);
IVS I/1 G/A	5'-TMR-CCTGGGCAGaTTGGTATCA-3'	(SEQ ID:50);
IVS I/6 T/C	5'-TMR-CCTGGGCAGGTTGtTATCA-3'	(SEQ ID:51);
IVS I/5 G/T	5'-TMR-CCTGGGCAGGTTGtTATCA-3'	(SEQ ID:52);
CD26 (N)	5'-TMR-GTTGGTGGTGAGGCCCTGG-3'	(SEQ ID:53);
CD26 G/A	5'-TMR-GTTGGTGGTaAGGCCCTGG-3'	(SEQ ID:54).

20. A customized oligonucleotide microchip for quantitation of the expression of a gene.

35   21. A customized oligonucleotide microchip for the detection of HLA polymorphism.

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22. The customized oligonucleotide microchip of
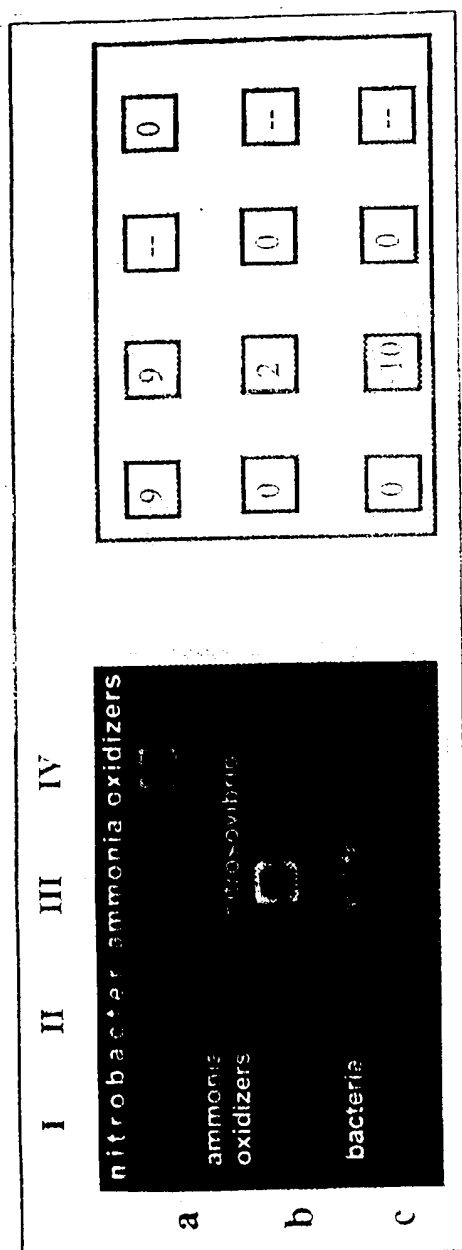claim 21 comprising the following oligonucleotides:

                    5'-AGGCAACGTG (SEQ ID:55);
                    5'-AGGCGACGTG (SEQ ID:56);
5   5'-GGTGAACTGG (SEQ ID:57);
                    5'-TAAATCTGCG (SEQ ID:58);
                    5'-AGGCAACATG (SEQ ID:59);
                    5'-CAAAACCTCC (SEQ ID:60);
                    5'-GCAAACACCA (SEQ ID:61);
10   5'-TACACCATAA (SEQ ID:62);
                    5'-ACTGCTCATC (SEQ ID:63);
                    5'-CAATGTCTTC (SEQ ID:64);
                    5'-CTCCTCATCT (SEQ ID:65);
                    5'-TGCCGGTCAA (SEQ ID:66);
15   5'-TTAGGACAGC (SEQ ID:67);
                    5'-ACACCACAAG (SEQ ID:68);
                    5'-CACAATGCCT (SEQ ID:69);
                    5'-CAGCAGTAGA (SEQ ID:70);
                    5'-TGCGGGTCAA (SEQ ID:71);
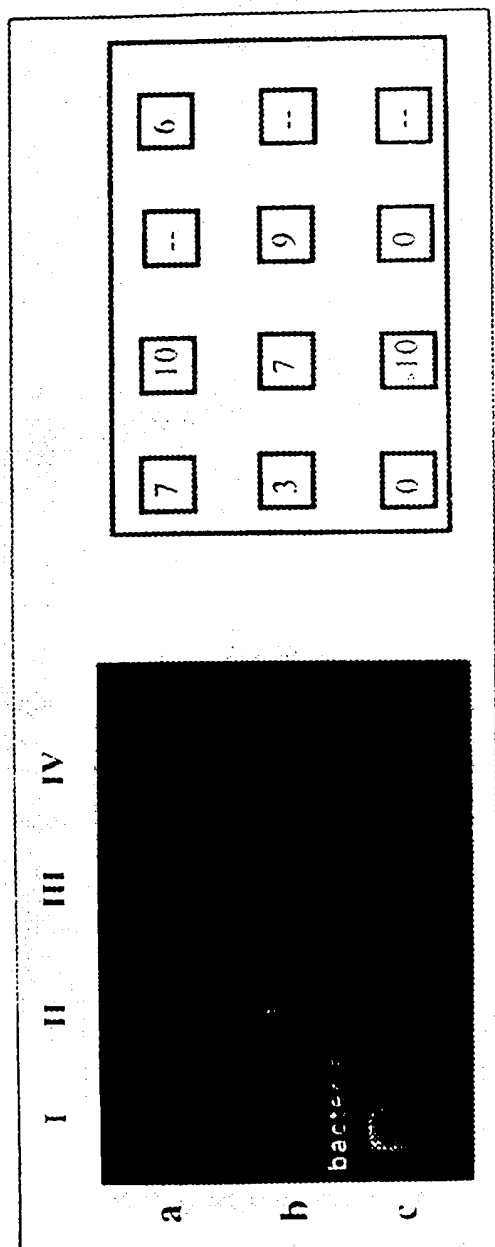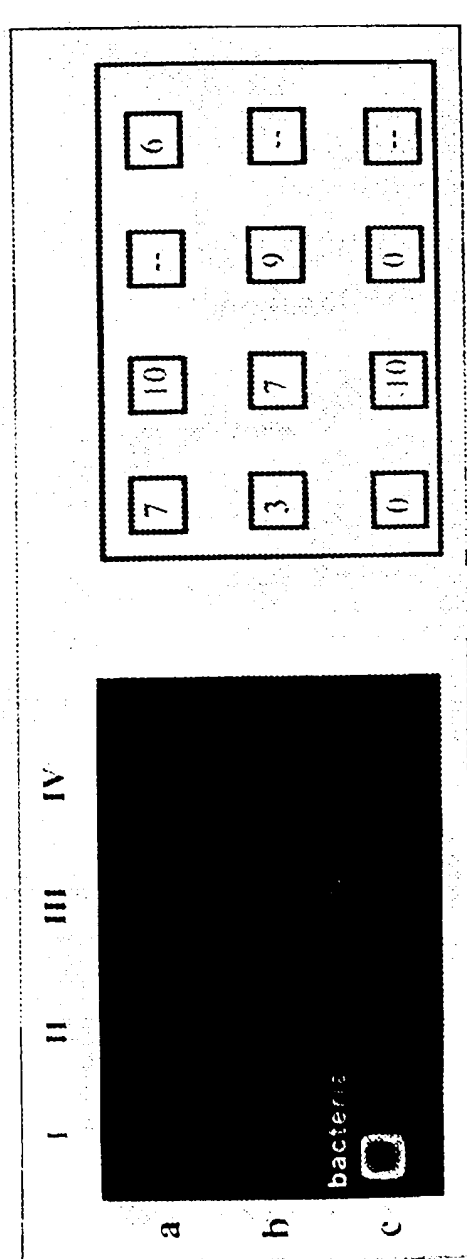20   5'-TTAGCACAGC (SEQ ID:72).

Fig. 1A

TEMPERATURE IN CENTIGRADE

Fig. 1B

TEMPERATURE IN CENTIGRADE
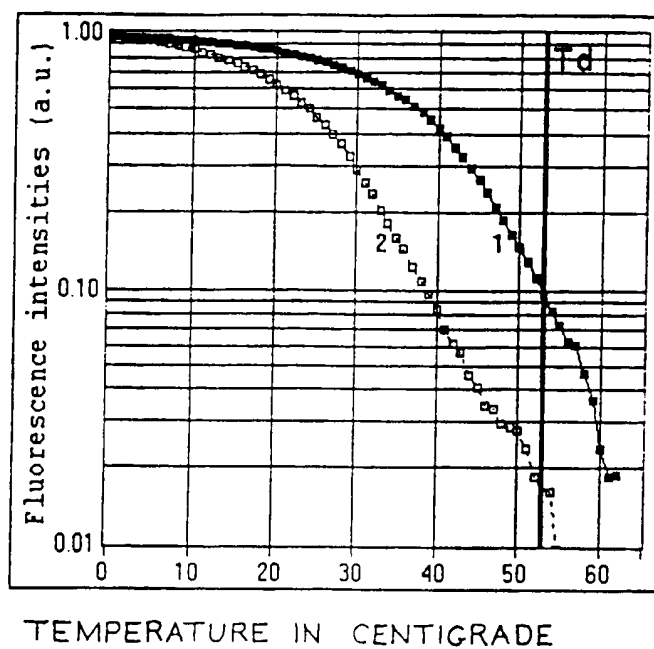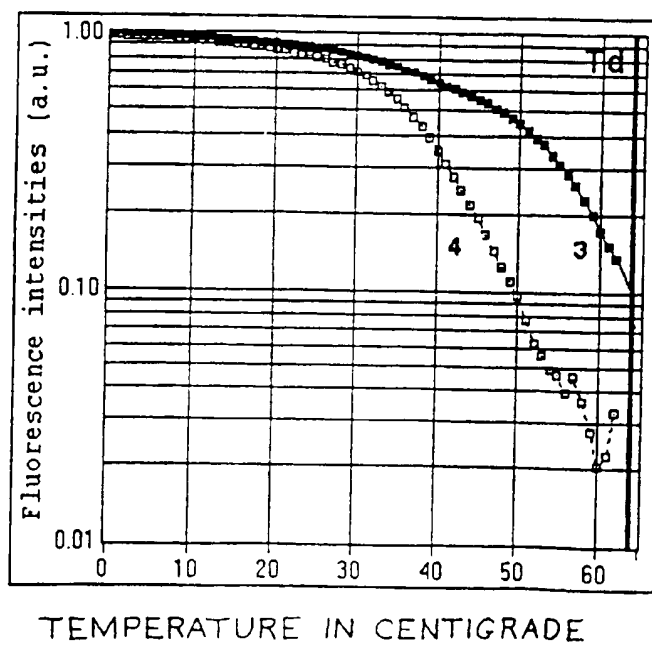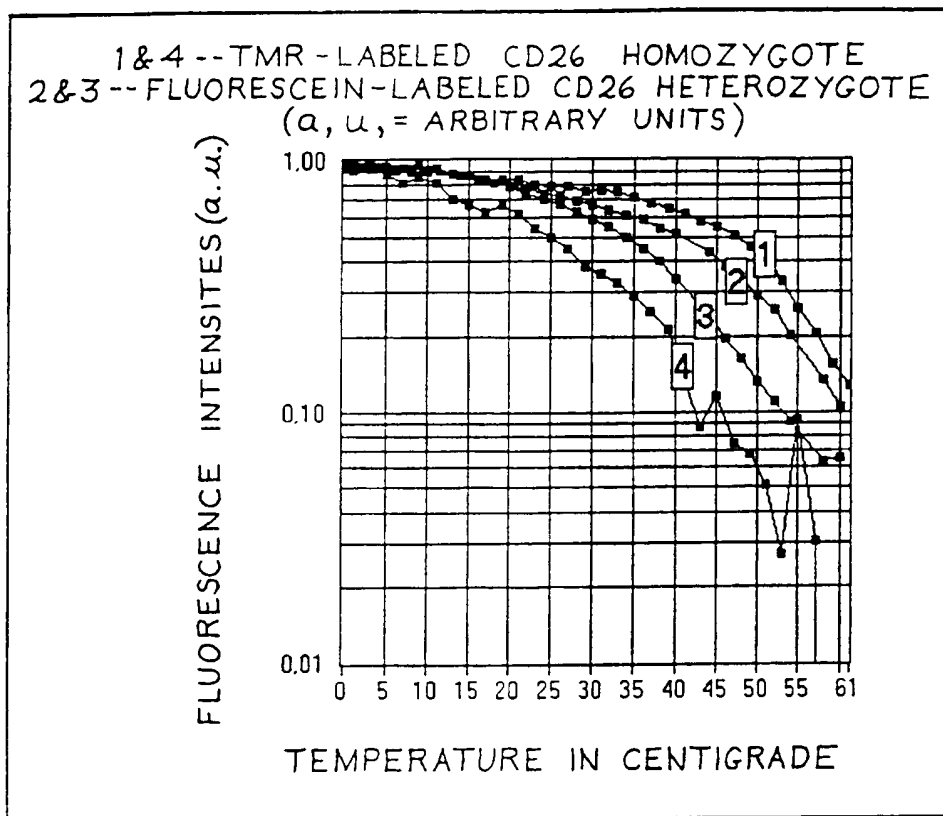
Fig. 2

Fig. 3A

Fig. 3B

Fig. 3c

Fig. 3d

Fig. 4A
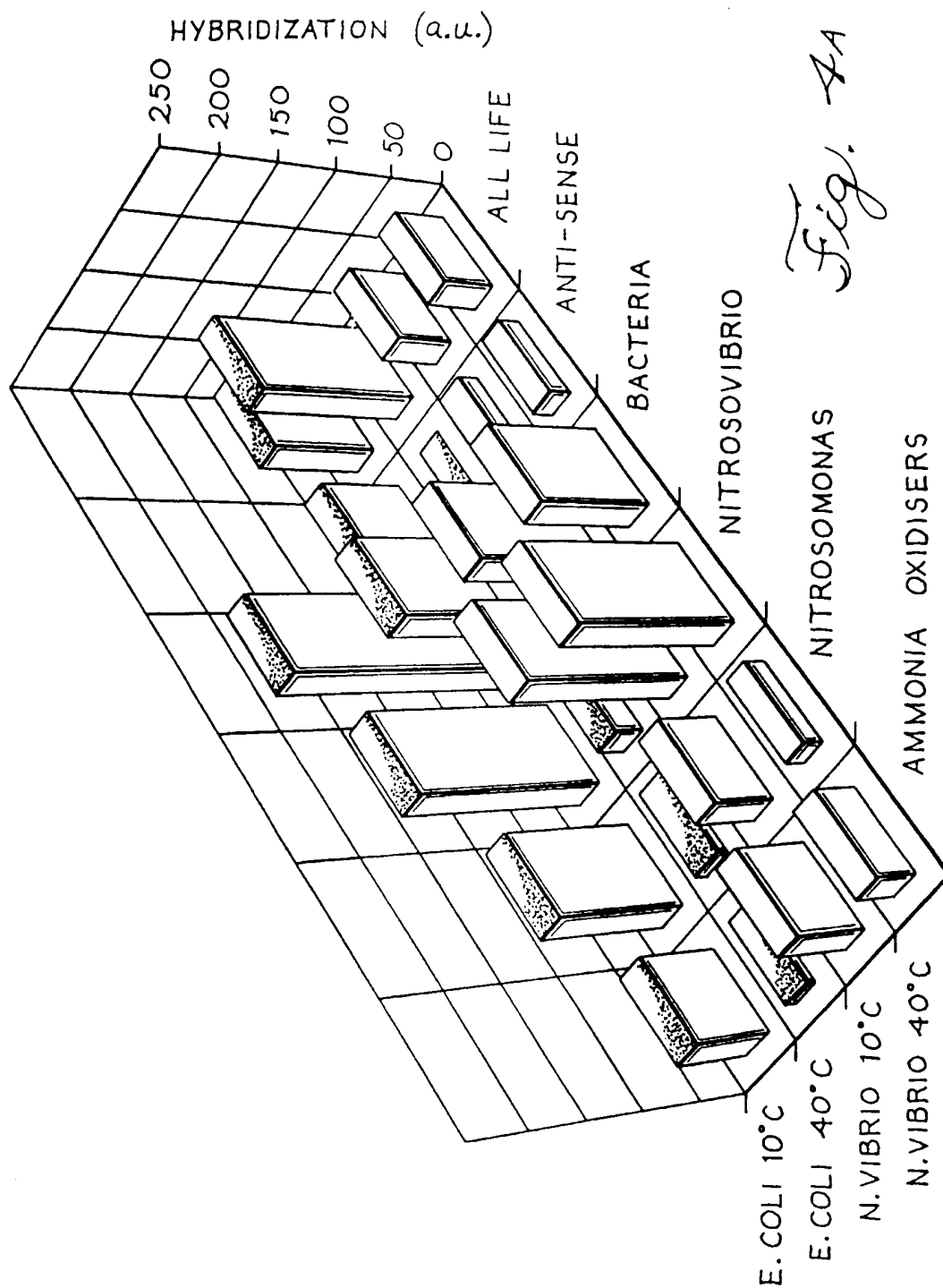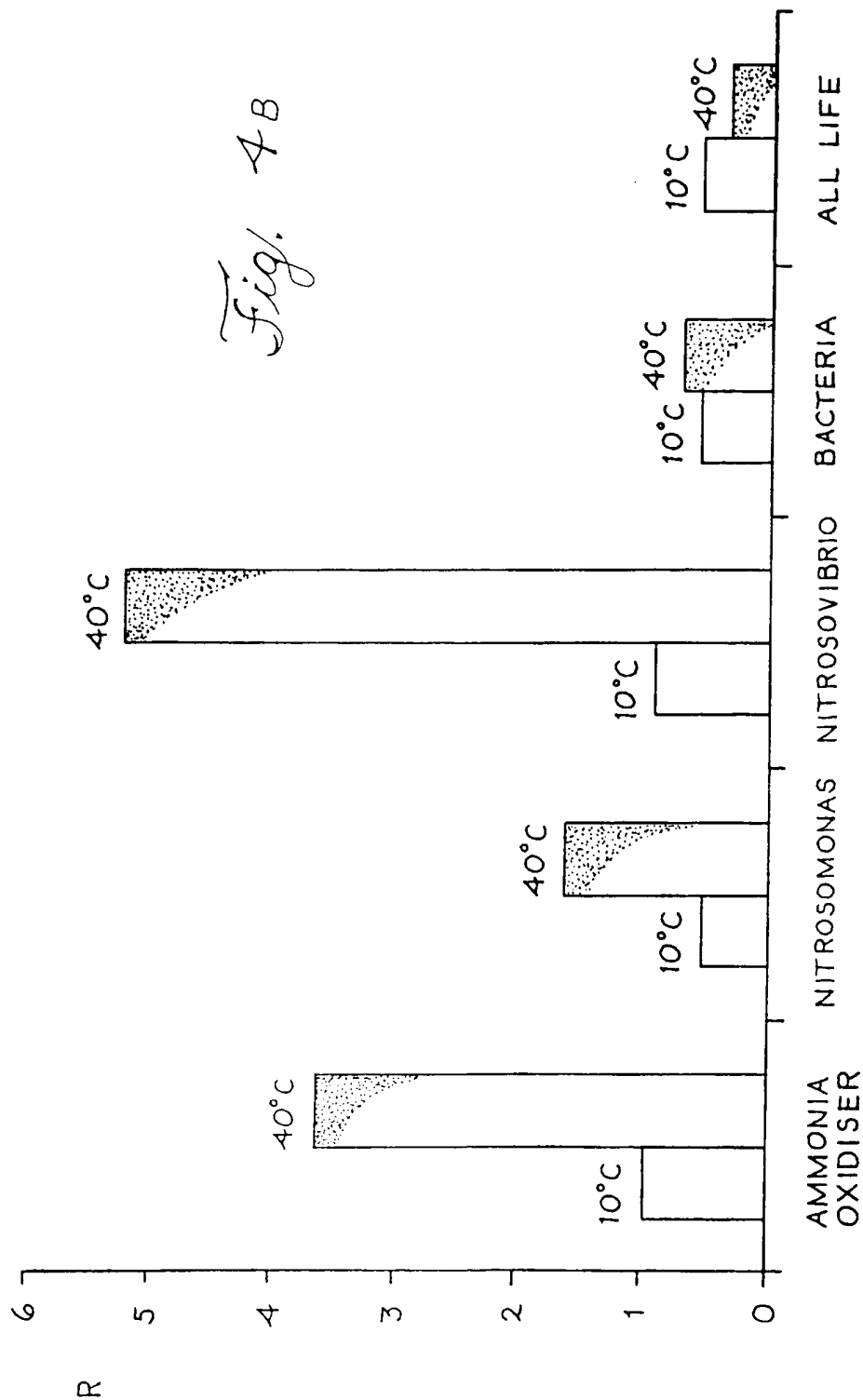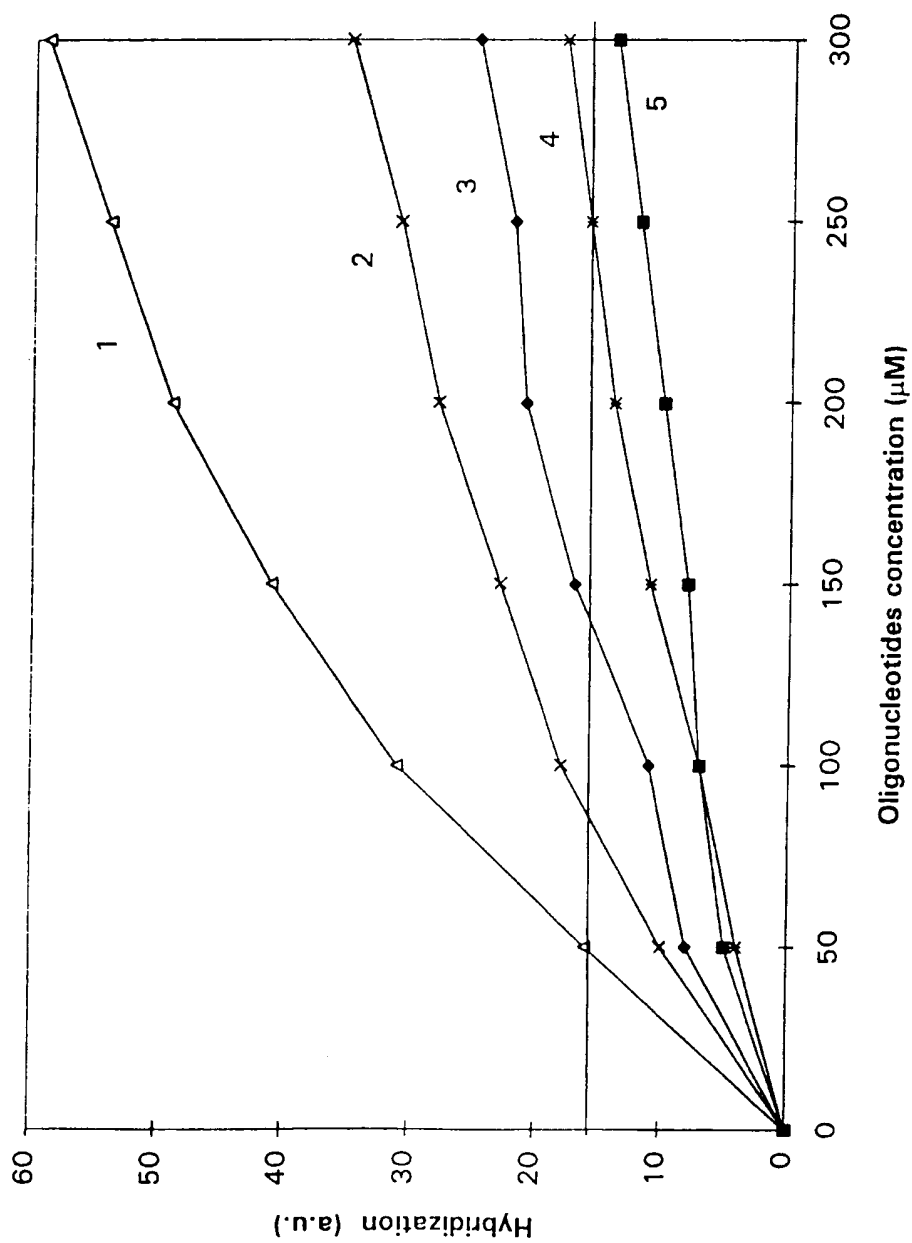
Fig. 4B
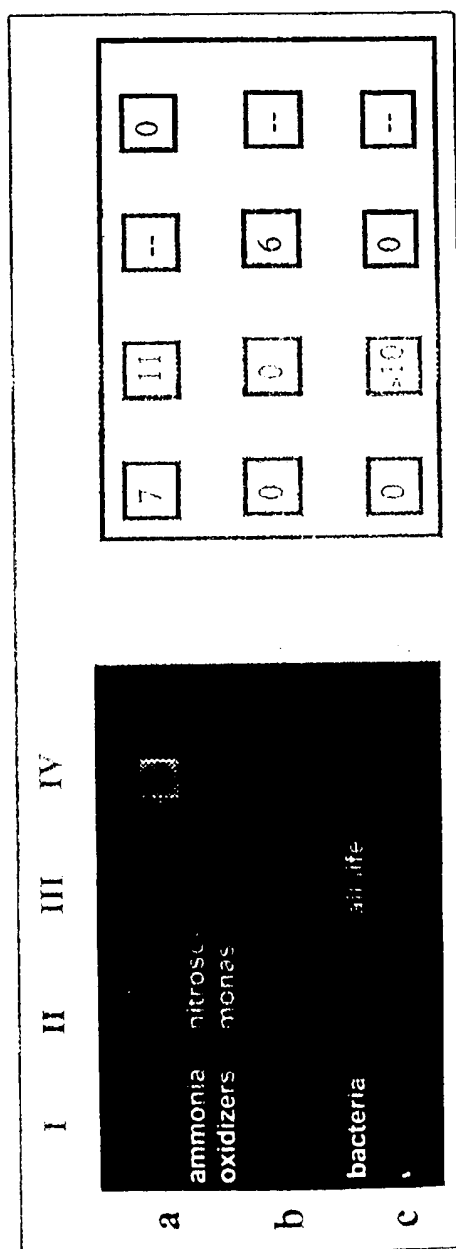
Fig. 5

a. IVS (N)   5'-TMR-CCTGGGCAGGTTGGTATCA-3'(SEQ ID NO:48);
b. IVS I/2 T/A   5'-TMR-CCTGGGCAGGaTGGTATCA-3'(SEQ ID NO:49);
c. IVS I/1 G/A   5'-TMR-CCTGGGCAGaTTGGTATCA-3'(SEQ ID NO:50);
d. IVS I/6 T/C   5'-TMR-CCTGGGCAGGTTGtTATCA-3'(SEQ ID NO:51);
e. IVS I/5 G/T   5'-TMR-CCTGGGCAGGTTGtTATCA-3'(SEQ ID NO:52);
f. CD26 (N)   5'-TMR-GTTGGTGGTGAGGCCCTGG-3'(SEQ ID NO:53);
g. CD26 G/A   5'-TMR-GTTGGTGGTaAGGCCCTGG-3'(SEQ ID NO:54);

Fig. 6

5' -AGGCAACGTG (1)(SEQ ID NO: 55);
5' -AGGCGACGTG (2)(SEQ ID NO: 56);
5' -GGTGAACTGG (3)(SEQ ID NO: 57);
5' -TAAATCTGCG (4)(SEQ ID NO: 58);
5' -AGGCAACATG (5)(SEQ ID NO: 59);
5' -CAAAACCTCC (6)(SEQ ID NO: 60);
5' -GCAAACACCA (7)(SEQ ID NO: 61);
5' -TACACCATAA (8)(SEQ ID NO: 62);
5' -ACTGCTCATC (9)(SEQ ID NO: 63);
5' -CAATGTCTTC (10)(SEQ ID NO: 64);
5' -CTCCTCATCT (11)(SEQ ID NO: 65);
5' -TGCCGGTCAA (12)(SEQ ID NO: 66);
5' -TTAGGACAGC (13)(SEQ ID NO: 67);
5' -ACACCACAAG (14)(SEQ ID NO: 68);
5' -CACAATGCCT (15)(SEQ ID NO: 69);
5' -CAGCAGTAGA (16)(SEQ ID NO: 70);
5' -TGCGGGTCAA (17)(SEQ ID NO: 71);
5' -TTAGCACAGC (18)(SEQ ID NO: 72);

Fig. 9

Fig. 7

SUBSTITUTE SHEET (RULE 26)

Fig. 8